THE
BIOLOGICAL BULLETIN

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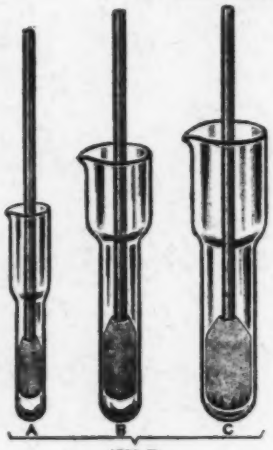

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Grinding vessel is made of precision bore Pyrex brand glass tubing with walls 2 mm thick and with expanded upper portion for overflow. Clearance between chamber and pestle is held to approximately 0.13 mm. Pestle is furnished with removable rod of either Stainless steel or aluminum. Pestles and grinding vessels of the same size can be used interchangeably and all components can be autoclaved.

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THE
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CARBOHYDRATE METABOLISM OF THE DEVELOPING EGG AND EMBRYO[1]

JOSEPH HALL BODINE AND WILLIAM LIONEL WEST

Zoology Laboratory, State University of Iowa, Iowa City, Iowa

Studies on the respiration of intact eggs and isolated embryos of the grasshopper, *Melanoplus differentialis,* reveal that the functional intensity of the enzymes concerned with aerobic metabolism is altered considerably during different stages of embryonic development (Bodine and Boell, 1934; Bodine and Boell, 1936; Boell, 1935; Bodine and Lu, 1950a, 1950b; Bodine, Lu and West, 1952). The question of the nature and relative amounts of various metabolites used during embryonic development has attracted the attention of many investigators using a diversity of biological materials (Spratt, 1952). Needham (1942) has summarized large amounts of data and introduced a concept of a definite sequence in which metabolites are used during the course of embryonic development. It is also generally accepted that the terrestrial cleidoic egg consumes large amounts of fat and a much smaller amount of protein and carbohydrate during development. Results previously recorded in this laboratory seem to support these above views (Boell, 1935; Hill, 1945). Much of the evidence pertaining to metabolism during embryonic development has been obtained from data on the intact egg and little from studies of the intact embryo freed of yolk.

The importance of carbohydrates as energy sources in biological systems is well known. The present investigation is the first of a series planned to determine the importance of carbohydrate in the mechanism of the gross chemical transformations from yolk to embryo in the developing egg of the grasshopper.

Methods

Embryos of the grasshopper, *Melanoplus differentialis,* were obtained, yolk free, by previously described methods of Bodine and Boell (1934, 1936).

Fractionation of the embryos (Bodine and Lu, 1950a, 1951) was carried out either in Ringer solution buffered with 1/15 *M* phosphates (pH 6.8) or in 0.25 *M* sucrose containing 0.0035 *M* magnesium and calcium chlorides. No quantitative differences in results were observed between the two media and the latter was the one of choice since the morphological integrity of the intracellular particles seemed

[1] Aided by a grant from the National Institutes of Health. Acknowledgment is gratefully made to Etta Andrews for technical assistance.

better preserved in it. The calcium ion in the sucrose medium was necessary to prevent clumping of nuclei and destruction of the nuclear membranes. The magnesium ion was found essential for maximum reactions of homogenates.

Respiration experiments were carried out using standard Warburg manometers. Seven experimental manometers contained 2,2,4-trimethyl pentane colored with Sudan IV as a manometer fluid and seven similar ones contained Brodie's solution. Both sets of manometers were carefully checked against each other. The sensitivity of the manometers was greatly increased by the use of the 2,2,4-trimethyl pentane as manometer fluid.

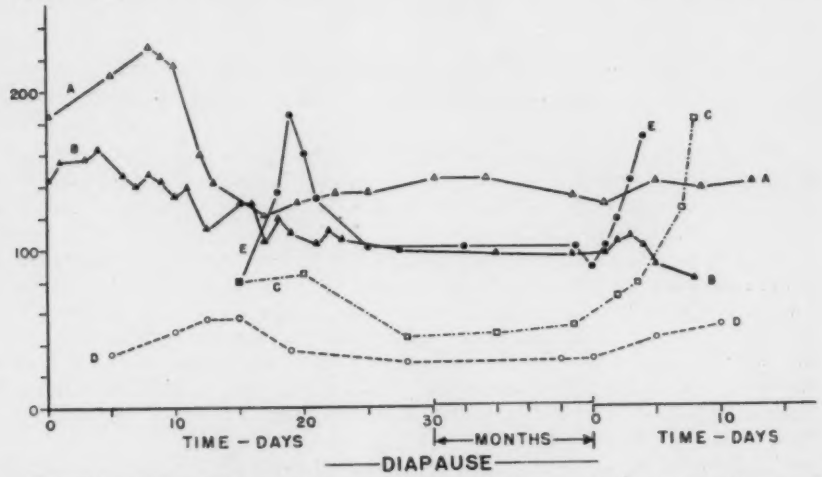

FIGURE 1. Shows total amount of carbohydrate per intact whole egg (A), and per yolk of whole egg (B); hexose content of embryo (C), and yolk (D), throughout course of embryonic development. Curve (E) shows endogenous O_2 uptake of embryo homogenates, taking that for the diapause embryo homogenate as 100%. Abscissa, time in days and months; ordinate micrograms per egg or embryo for carbohydrates, for O_2 uptake, percentage. Values for hexoses of embryo are increased by 1/10 over actual values found. Each point represents averages from many experiments.

Commercial glucose, glucose-1-phosphate (dipotassium salt), fructose-6-phosphate (dibarium salt converted to the sodium or potassium salt) and fructose 1,6-diphosphate (dibarium salt converted to sodium salt) obtained from the Swartz laboratories, were employed as substrates. The substrates (0.5 cc.) were tipped from the side arms of the manometer flasks at the end of a 40-minute control period. The final concentration of the substrates per 1.5 ml. (total volume of fluid in flask) was approximately 40 moles $\cong$ 0.026 *M* for glucose, while fructose 1,6-diphosphate contained the same carbohydrate content per cc. as did glucose-1-phosphate.

Carbohydrate determinations were made with the commercial product "Microne" obtained from the National Biochemical Laboratory. "Microne" is chemically anthrone and was described by Dreywood (1946) as a specific test for carbohydrate. The maximum absorption of the green color developed using a glucose test solution

and 0.2% "Microne" in 95% sulfuric acid was found to be 625 mμ which is similar to that reported by Viles and Silverman (1949). "Microne" is used as a microcolorimetric method for determination of sugars, starches and celluloses. This reagent is of interest since equivalent amounts of glucose, glycogen and fructose

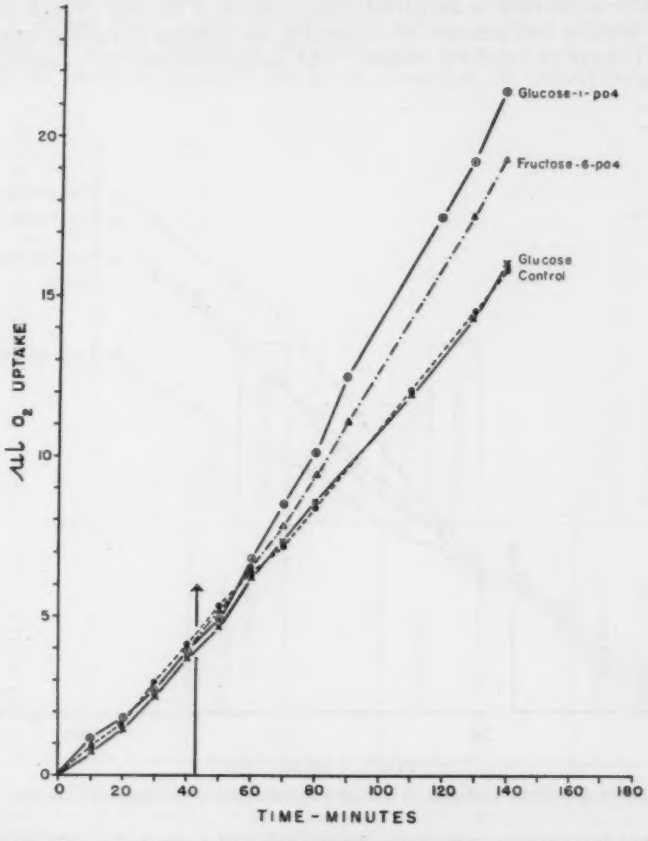

FIGURE 2. Shows endogenous O_2 uptake of diapause embryo suspended in different substrates. Arrow indicates addition of substrate. Abscissa, time in minutes; ordinate, micro liters of O_2 per egg. Graphs represent typical experiment.

give approximately the same amount (intensity) of color with the reagent (Morse, 1947; Morris, 1948).

To 3 cc. of an experimental solution, 6 cc. of "Microne" were added from a burette that permitted rapid flow of the viscous acid solution. The components of the tube were mixed rapidly and placed in a water bath (25° C. ± 0.2) 30 minutes for color development. At the end of this period, measurements were made imme-

diately using the Coleman Model IU spectrophotometer at 625 mμ. A series of standards was run with each group of experimental solutions.

Analyses were made on each of the following: (a) An acid hydrolysis of the whole egg which is recorded as the total carbohydrate (Fig. 1, curve A). These data are strikingly similar to those found by Hill (1945). (b) A saline-soluble fraction which is recorded as the carbohydrate content of the yolk (Fig. 1, curve B). This latter fraction was obtained by suspending the contents of the egg in a concentration of 1 egg/2 cc., in 0.9% saline. The suspension was then centrifuged for

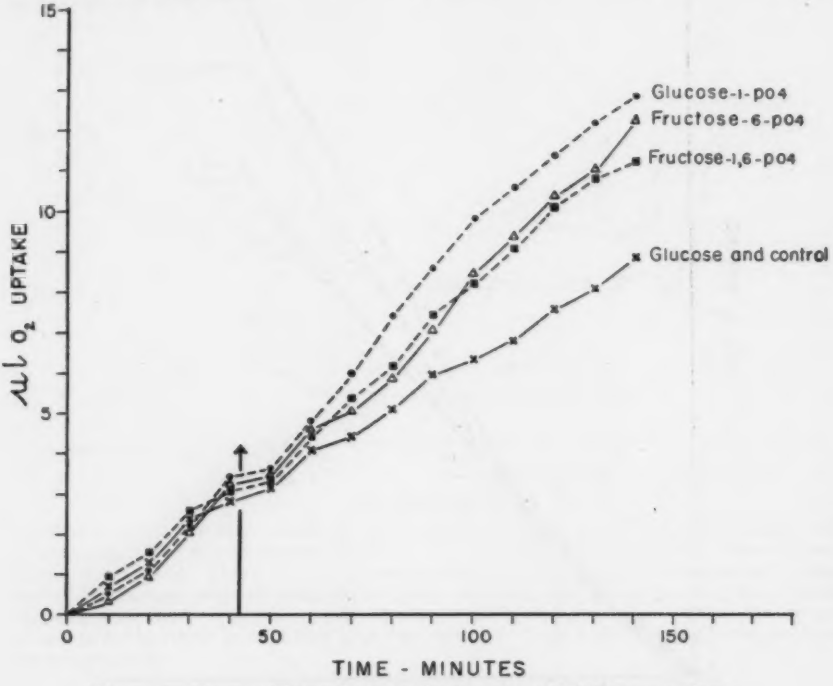

FIGURE 3. Same as Figure 2 except for homogenate of diapause embryo.

one minute at low speed to remove embryo, shell and extra-embryonic membranes, after which an aliquot was taken for analysis. (c) A trichloracetic acid (TCA)-soluble fraction which is recorded separately as the hexose content of the embryo and the yolk (Fig. 1, curves C and D, respectively). The yolk hexose was obtained by breaking the eggs in 10% TCA and then removing the intact embryos, precipitated protein and shells by centrifugation (5000 G). Lipids were removed from centrifuged samples by means of suction pipettes. An aliquot of the supernatant was diluted and used for analysis and designated as yolk hexose. The embryo hexose was determined by homogenization of saline-washed embryos in 10% TCA. Centrifugation (5000 G) was employed to remove the precipitated protein and

suction pipettes to remove the lipid layer. An aliquot was then diluted and used for analysis.

Results

The total carbohydrate content of the whole egg is graphically represented in Figure 1, A and these values are strikingly similar to those reported earlier by Hill (1945). An examination of this curve shows a marked initial increase during pre-diapause, which is probably due to the formation of carbohydrate-containing

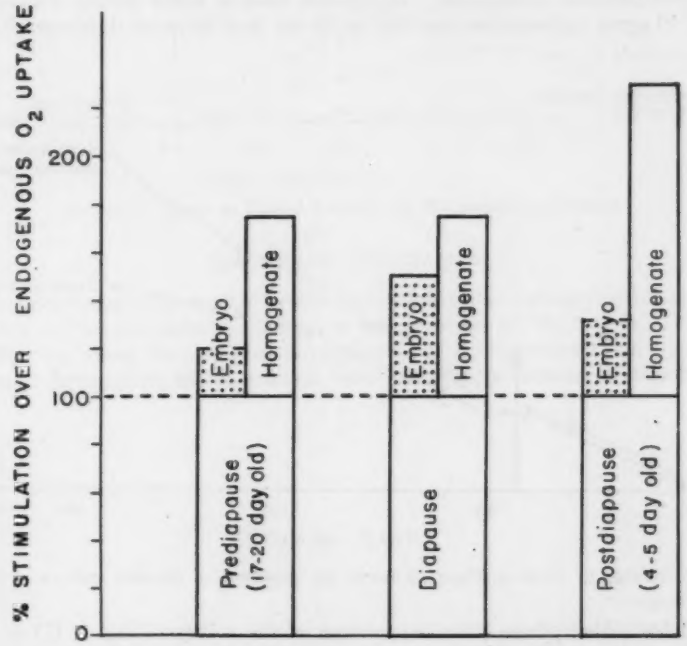

FIGURE 4. Shows percentage stimulation over the endogenous O_2 uptake of intact embryos and homogenates due to addition of substrates, glucose-1-PO_4, fructose-6-PO_4, in final concentration of 0.026 *M*. Stages of development indicated on abscissa; per cent stimulation on ordinate. Data taken from averages of all experiments.

cuticles. This increase is followed by a drop in total carbohydrate to a value which remains rather constant throughout the remainder of embryonic development. The general significance of the carbohydrate content of the whole egg for development has been discussed by Hill (1945) and no further reference to it will be made at this time.

The concentration of carbohydrates in the yolk is graphically presented in Figure 1, B. An examination of this curve shows that from the time of laying to about the fifth day pre-diapause, there appears to be an increase of approximately

19 μgms. of carbohydrate per egg. This rise is followed by a fall of approximately 40 μgms. carbohydrate per egg between the fifth and fifteenth days of pre-diapause development. This latter concentration is maintained throughout the remaining pre-diapause and diapause developmental periods but falls again during post-diapause (Fig. 1, B).

The concentration of the TCA-soluble carbohydrate of the yolk (Fig. 1, D) is considered to represent the hexoses. These concentrations are similar to the free reducing substances of Hill, who also suggested them to be hexoses. An analysis of curve D reveals a drop of approximately 32 μgm. carbohydrate per egg from 15-day pre-diapause to diapause. A constant level is noted during diapause and a rise of 20 μgms. carbohydrate per egg by 10-day post-diapause development. The

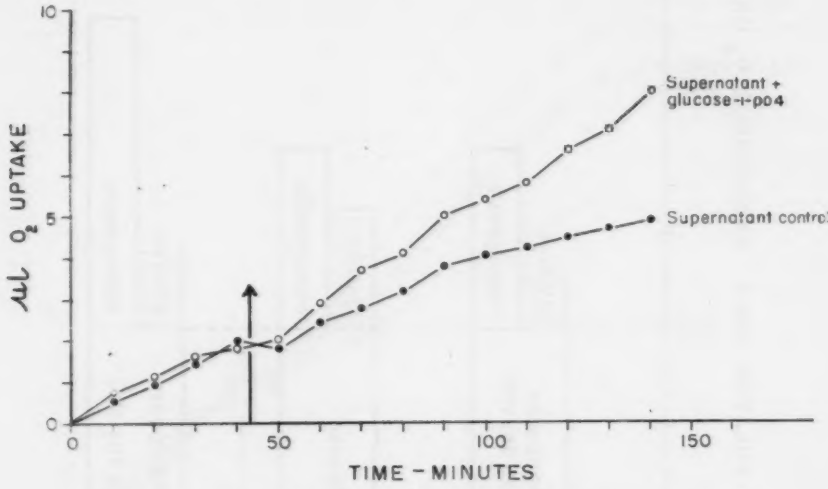

FIGURE 5. Same as Figure 2 except for cytoplasm of diapause embryo.

TCA-soluble carbohydrate (hexose) content of the embryo (Fig. 1, C) is higher in both pre-diapause and post-diapause than in diapause. This result agrees qualitatively with that presented by Hill (1945). The values recorded here, however, are approximately one-tenth of those presented by him for the free reducing substances of the embryo.

RESPIRATION OF THE ISOLATED, INTACT EMBRYO

Embryonic respiration during the mitotically active and blocked stages of development is stimulated by the addition of hexose monophosphates while glucose has no effect over a 100-minute exposure (Figs. 2 and 4). The percentage stimulation due to the hexose phosphates during diapause or blocked periods appears greater than that in pre- and post-diapause (Fig. 4). Hexose diphosphate, although not extensively investigated, seems to act similarly to hexose monophosphates.

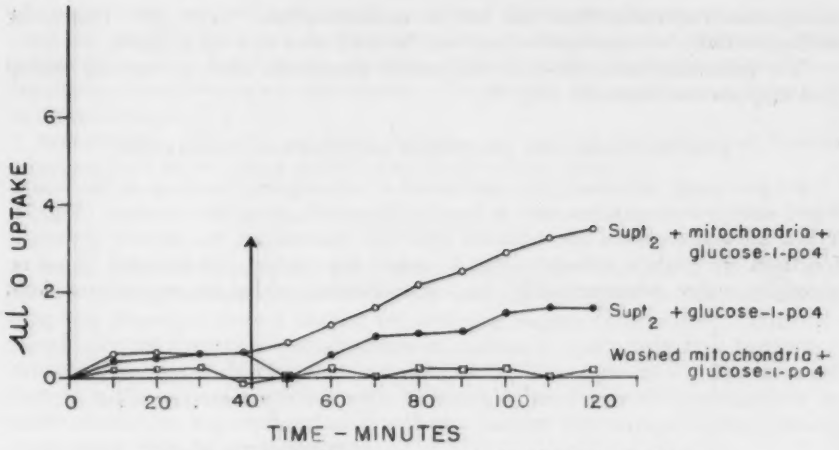

FIGURE 6. Same as Figure 2 except for fractionated cytoplasm.

HOMOGENATE RESPIRATION

The endogenous respiration of broken embryonic cells (homogenate) is markedly stimulated by hexose monophosphates in the presence of Mg^{++} (Fig. 3). Mg^{++} alone does not affect the endogenous respiration of homogenates (Fig. 3).

Hexose diphosphate shows a much lower percentage increase of stimulation of

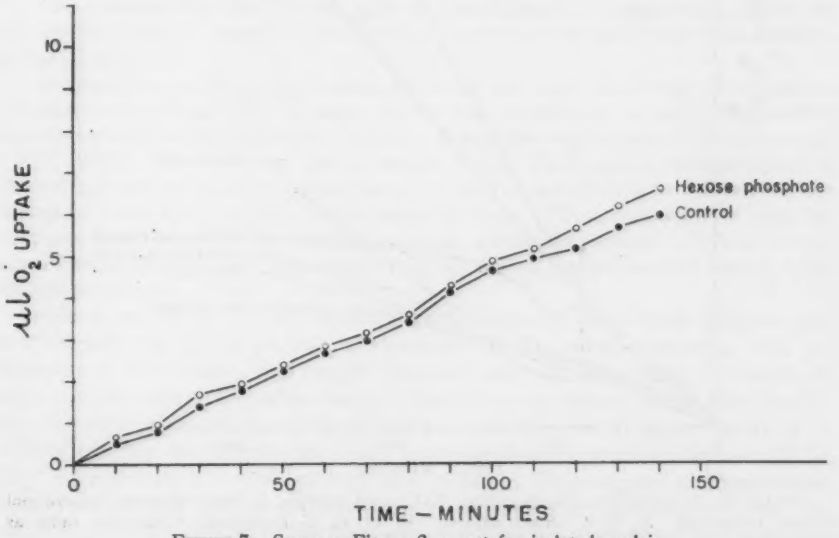

FIGURE 7. Same as Figure 2 except for isolated nuclei.

endogenous respiration than the hexose monophosphates (Fig. 3). This weak aldolase activity was unexpected and will be dealt with in a later paper.

The percentage stimulation of endogenous respiration tends to increase during post-diapause development (Fig. 4).

Fractionation and Inhibition of Cellular Respiration

As previously indicated, the supernatant or cytoplasmic fractions of the centrifuged embryo homogenates seem to contain the aerobic glycolytic enzymes (Fig. 5). When the mitochondria are removed from the supernatant the aerobic glycolytic functions are slightly reduced (Fig. 6) while the washed mitochondria show no glycolytic action whatever (Fig. 6). Mitochondria added to supernatant from

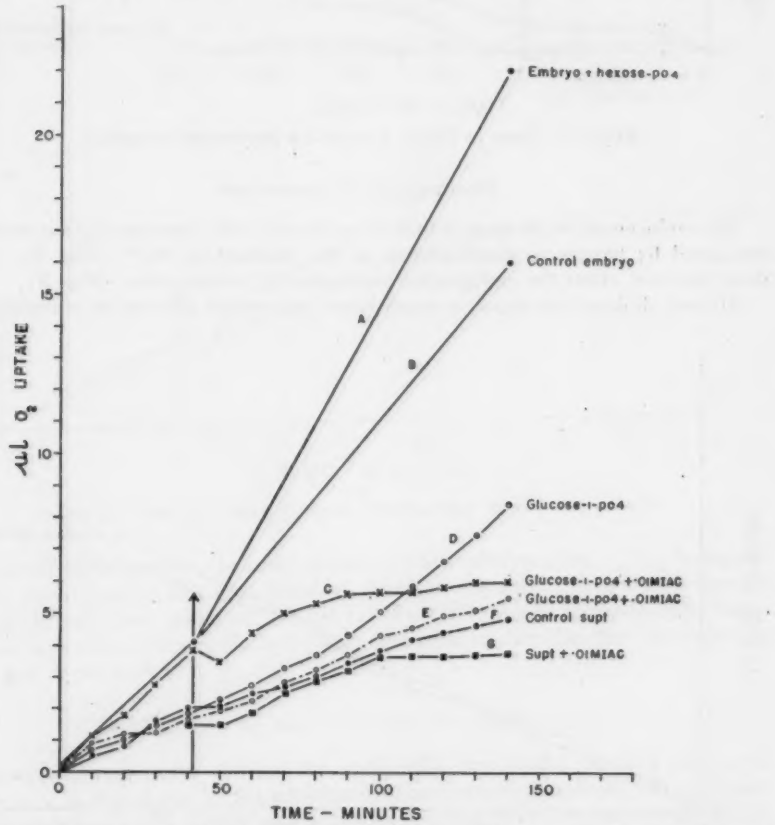

Figure 8. Shows effect of iodoacetate (IAC) on O_2 uptake of intact diapause embryo and isolated cytoplasm. A, B, C intact embryo; D, E, F, G cytoplasm. Otherwise same as Figure 2.

which they were removed tend to show an added action over the supernatant alone. Once the original fraction is broken down into its various constituents and these in turn again added together, the glycolytic action (aerobic) is greatly reduced over that of the non-fractionated supernatant. No aerobic glycolytic action occurs in the isolated nuclei (Fig. 7).

Iodo-acetate (0.01 *M*) markedly inhibits the aerobic metabolism of hexose phosphate both in the intact embryo and its cytoplasm (Fig. 8).

Discussion

Some chemical changes in carbohydrates during development of the egg and embryo of the grasshopper have been previously investigated in this laboratory, using the Hagedorn-Jensen method for reducing sugars (Hill, 1945). Hill discussed fully the changes in carbohydrates in relation to extra-embryonic membrane formation, embryonic development and energetics. He suggested that the chemical changes in the egg are consistent with Needham's theory of a definite sequence in which metabolites are used and also with the concept that terrestrial eggs during development burn fat predominantly.

It is apparent from the present results that an initial fall in carbohydrate content of the yolk between 0-day and 15-day pre-diapause is followed by no marked change in the yolk until the post-diapause period. The curve for total carbohydrates (Fig. 1, A) also reveals qualitatively similar results. Hill associates these early (pre-diapause) and late (post-diapause) changes with the carbohydrate phase of metabolism and extra-embryonic membrane formation which probably involves protein metabolism, and also suggests that the increased carbohydrates may be in part supplied by conversions of protein.

The carbohydrate lost by the yolk in post-diapause is apparently gained by the embryo since a constant carbohydrate content in the acid hydrolysis fraction exists (Fig. 1, D).

A comparison of the oxygen consumed during the stages 18–20-day pre-diapause, diapause and 4–5-day post-diapause shows that changes in carbohydrate content cannot account for the increased intensity of aerobic metabolism of the intact egg (Hill, 1945). However, the loss in hexoses (10% TCH-soluble carbohydrate) of the embryo and yolk during pre-diapause is more than sufficient to account for the increased respiratory activity of the intact embryo. Thus, carbohydrate may act as a source of energy for the marked synthesis within the embryo. This is further supported by data on the R.Q. which is slightly higher for the isolated embryo than for the intact egg.

Bodine and Boell (1934) observed that mitotically active post-diapause embryonic cells can utilize glucose, a stimulation in O_2 uptake occurring after an exposure of two hours. The results presented here for 100 minutes exposure do show that the embryo can utilize hexose phosphates to a greater extent than glucose over the same length of time (Figs. 2 and 4). Phosphorylated hexose seems to be utilized whether the cells are mitotically active or blocked, intact or broken. (The Mg^{++} ion is necessary for utilization by broken cells.) This suggests that hexokinase activity is either absent or present in very small amounts as compared with the enzymes necessary for the aerobic breakdown of these phosphorylated intermediates.

The apparent absence of hexokinase is paralleled by a lack of alkaline phosphatase activity in the embryo until approximately 8 days post-diapause. Fitzgerald (1949) suggested that alkaline phosphatase might be concerned in the transport of food material to the embryo, since consistent increase in phosphate in both pre- and post-diapause is paralleled by an increased alkaline phosphatase activity of the yolk.

The free reducing substances of Hill and the present TCH-soluble carbohydrate (Fig. 1) are a possible source of hexose, with phosphorylation perhaps occurring within the yolk itself or at the cell membrane of the embryo.

The percentage stimulation of embryonic respiration using hexose monophosphates as substrate varies markedly over the stages investigated (Fig. 4). It is greatest in diapause. However, the percentage stimulation of the homogenate does not parallel that of the embryo either qualitatively or quantitatively, being greatest in mitotically active post-diapause. This may indicate that diffusion is a limiting factor, particularly since the concentration of TCA-soluble carbohydrate is higher in 17–20-day pre-diapause and 4–5-day post-diapause than that in diapause. Further, the maximal increase in homogenate stimulation is similar for both diapause (blocked) and pre-diapause (active) while the embryonic stimulation varies, indicating perhaps a higher relative saturation of the enzyme in the intact mitotically active embryos. Moreover, the constancy of stimulation of the homogenate in pre-diapause and diapause indicates little, if any, synthesis of enzymes associated with aerobic glycolysis during this developmental period. Either a marked synthesis or increase in activity must occur at post-diapause, as shown by the great increase in stimulation of the homogenate.

Following separation of the embryo homogenate into supernatant and nuclear fractions, the enzymatic activity was found to reside primarily in the supernatant. The mitochondria (large granules) isolated from the supernatant by centrifugation were not stimulated by added hexose phosphates. The supernatant (microsomes and remaining cytoplasm) shows very weak stimulation, while a combination of the two fractions gives a higher percentage O_2 uptake but not equivalent to the original supernatant itself. Thus, the enzymes associated with aerobic glycolysis appear more intimately associated with the soluble protein fraction, as has been shown to be the case for mammalian material.

Iodoacetate, a dehydrogenase inhibitor, believed by some to be specific for aldolase activity, inhibits almost completely the stimulation of the hexose monophosphates. A recent article by Cleland and Rothschild on the oxidation of carbohydrate of the sea-urchin egg strongly suggests metabolic changes in this material similar to those for the grasshopper embryo (Cleland and Rothschild, 1952).

Summary

1. A study has been made of the carbohydrate content and changes in the intact egg and isolated embryo of the grasshopper, *Melanoplus differentialis*, during the course of embryonic development.

2. Changes in the carbohydrate content of yolk have been compared with similar changes in the developing embryo.

3. Respiration of intact isolated embryos in different carbohydrate substrates indicates a marked utilization of phosphorylated compounds.

4. The endogenous oxygen uptake of embryo homogenates is markedly stimulated by hexose monophosphates in the presence of Mg^{++}.

5. Aerobic glycolysis seems to be controlled by enzymes located only in the cytoplasm of the embryonic cells.

6. Phosphorylation of carbohydrates occurs either in the yolk or at the surface of the embryonic cells.

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INDICATOR GRADIENT PATTERNS IN OÖCYTES AND EARLY DEVELOPMENTAL STAGES OF ECHINODERMS: A REEXAMINATION

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The gradient patterns, as observed by means of intracellular reduction of methylene blue and diazine green (Janus green), following staining by oxidized dyes were recorded for cleavage and later stages of the echinoids, *Strongylocentrotus purpuratus, S. franciscanus, Dendraster excentricus* and for the asteroid starfish, *Patiria miniata,* and for exogastrulae of these forms in earlier papers (Child, 1936a, 1936b). Later the pattern of the intracellular indophenol reaction during larval development of *Dendraster* was determined, together with some data on patterns of exogastrulae (Child, 1941). In these observations on indophenol pattern, extremely low concentrations of reagents were used so that the patterns became visible in living animals. Still later, data concerning the indophenol gradient patterns of *Patiria miniata* from the later ovarian oöcytes to larval stages, also with very low concentrations of reagents, appeared (Child, 1944).

However, intracellular oxidation of reduced dyes and of indophenol reagents has been found more satisfactory than reduction after staining in the study of gradient patterns, for reasons noted in the following section. The present paper is primarily concerned with new data concernjng gradient pattern, as determined by intracellular oxidation of indicators in stages from early ovarian oöcytes through embryonic development to larval stages of certain echinoderms.

Material and Methods

Two echinoids, *Strongylocentrotus purpuratus* and *Dendraster excentricus,* and the asteroid starfish, *Patiria miniata,* have served as material. The data include stages from the early ovarian oöcytes to earlier larvae of the echinoids. As regards the starfish, they are merely supplementary to the earlier study of the indophenol reaction in this form (Child, 1944). For the two echinoids these new data on intracellular oxidation of indicators constitute a more complete survey of indicator patterns than earlier studies and render differentials more clearly visible, particularly in certain stages. Deep indebtedness to the Director and staff of the Hopkins Marine Laboratory of Pacific Grove for collecting material, providing facilities for its use and, in various cases, for transporting it to Palo Alto, is gratefully acknowledged. For fertilization and development at Palo Alto filtered sea water kept in darkness has been found satisfactory.

In the first studies of indicator patterns in early echinoderm development (Child, 1936a, 1936b), the material was stained by an oxidized redox dye and reduction was brought about by oxygen uptake of embryos and larvae sealed in a small volume

of water or, in some cases, of dye solution. The decrease in free oxygen content of the solution brought about intracellular reduction of the dye. Reduction under these conditions was very slow and uniform distribution of oxygen was provided for by frequent change of position of the sealed material in relation to gravity or with motile stages by swimming activities. Reducing agents which had been used in earlier indicator studies were highly toxic and might themselves retard or inhibit reduction differentially by their toxicity.

As indicator studies continued it became increasingly evident that intracellular oxidized dyes, with increase in intracellular concentration, might also retard or even inhibit intracellular reduction differentially. This retardation or inhibition occurred most rapidly in the regions which reduced most rapidly in uninjured material; *i.e.*, if the oxidized indicator attained a certain intracellular concentration it might decrease or even obliterate slight regional differentials of pattern in consequence of the differential susceptibility of different gradient levels to the dyes, particularly the more toxic dyes such as diazine green (Janus green). In some cases a gradient pattern has become reversed in direction by the differential retardation or inhibition of intracellular reduction. In use of reduced dye solutions for intracellular oxidation, the toxic reducing agents might alter gradient pattern before intracellular oxidation began.

These difficulties are almost entirely avoided by use of sodium hydrosulphite as a reducing agent, either for dye solutions to be used for intracellular oxidation or for intracellular reduction after staining by an oxidized dye. This agent is not appreciably toxic, even with hours of exposure to concentrations far above those required to reduce the indicators, either in solution externally or intracellular. Very small quantities of this agent reduce dye solutions in a few seconds and it can be used at once for intracellular oxidation or for intracellular reduction of material previously stained by an oxidized dye. In the present paper it is used primarily for intracellular oxidation of dyes. A few minute granules of hydrosulphite, less than a milligram, are sufficient to reduce completely one ml. of methylene blue or diazine green solution and various other dyes in concentrations found useful. Intracellular oxidation occurs within a few minutes without evidence of toxic effect until intracellular dye concentration becomes high. Care must be taken not to use an excess of hydrosulphite beyond that required to reduce the dye. Any considerable excess may retard or even prevent intracellular oxidation in consequence of too complete removal of oxygen. After intracellular oxidation addition of a little more hydrosulphite will result in differential intracellular reduction. In many organisms differential oxidation and reduction can be repeated without alteration of pattern. These methods of using sodium hydrosulphite, although noted in earlier papers, are described in detail here because their use on early stages of echinoderm development is regarded as a somewhat critical case near the lower limit of availability of redox dyes for rendering visible regional differentials of pattern. Intracellular oxidation from reduced dye solutions has been called for convenience primary oxidation in order to distinguish it from re-oxidation, following reduction.

Redox dyes used for intracellular oxidation, often with following reduction, were methylene blue in various concentrations from 1/50,000 to 1/20,000, diazine green from 1/100,000 to 1/50,000 and Nile blue sulphate 1/100,000. These were

used chiefly on *S. purpuratus* for comparison with the indophenol reaction; on *Dendraster* and *Patiria* the indophenol reaction was used almost entirely. Primary oxidation of the redox dyes and the intracellular oxidation of the indophenol reagents have made visible the same patterns, though in certain cases the indophenol reaction has shown the differential more distinctly than the dyes.

The indophenol or Nadi reaction, the intracellular formation of the deep blue indophenol from para-aminodimethylaniline (dimethyl-paraphenylene diamine) and α-naphthol, catalyzed by an oxidase, "indophenol oxidase," commonly regarded as cytochrome oxidase or a related enzyme, has been found extremely useful in the studies of echinoderm pattern and in many other organisms. In several earlier papers the modifications of the reaction found most useful in rendering visible the slight differentials of early developmental stages and small single cells have been described. However, since presence of a gradient pattern in early stages of echinoderm development has been questiond, it has seemed necessary to call attention once more in some detail to the method, as used with low concentrations of reagents. Also certain points not particularly considered earlier are noted.

The aniline has been obtained in 10 gram amounts from the Eastman Kodak Co. Some of these have been liquid, others solid at room temperature and, according to information from the Kodak Co. laboratories, the solid form is more nearly pure. However, the melting point is only slightly above room temperature and immersion of the bottle containing the aniline in slightly warmed water liquefies it. Since the aniline is volatile and an acrid, highly irritating poison, weighing of the very small amounts required presents difficulties, and since earlier use of the aniline was in terms of small drops of the liquid, its use has been continued in this way. Although attempts have been made in earlier papers to give information concerning concentrations used, the actual concentrations are not known in any particular case and since the same pattern of reaction appears with a very wide range of concentrations and the method is not quantitative, the chief point at present is use of concentrations found by experience to give the reaction with as little indication of toxicity as possible. For most organisms and even for many single cells a solution, consisting of one small drop (30–40 = 1 ml.) in 10 ml. of salt or fresh water, according to the material, is made daily or oftener; the aniline gradually oxidizes in water. The concentration of this solution can be altered as desired. This is essentially a stock solution and is diluted for use. The α-naphthol stock solution consists of one mg. or less, estimated after repeated weighings as a basis for estimation, in 10 ml. of water, salt or fresh. Enough naphthol dissolves within a few minutes to give the reaction readily without addition of KOH or NaOH necessary to dissolve the naphthol when high concentrations of the agents are used.

The solution for use, regarded as standard, merely because it provides a starting point for determining the most satisfactory solution for a particular material, consists of one drop each of the aniline and the naphthol stock solutions in one ml. of water. The naphthol is much less toxic than the aniline and can be used in considerably higher concentrations if desired, but in any case it is not certain just how much of the naphthol dissolves without addition of alkali. In some organisms and with some cells concentrations half or a fourth of the standard, or even less, are desirable. Solutions four to five times the standard are usually rather rapidly toxic. In general the practice found most effective has been to use the lowest concentrations of reagents which render the intracellular reaction clearly visible within a

reasonable period of observation, *i.e.*, 10–30 minutes, although it is often possible to obtain a very distinct differential reaction in four to five minutes. The method is of course at present far from quantitative; its use in any case is a matter of trial and error, but it does render directly visible certain characteristics of physiological pattern in living organisms which are not now directly distinguishable in any other way, except with the method described above with redox dyes. With these general ranges of concentration and variations from them as occasion required, the indophenol reaction has made directly visible patterns of oxidase activity in numerous organisms from protozoa to vertebrates. At least the earlier stages of the reaction occur in apparently uninjured individuals; motile forms continue activity and non-motile developmental stages or forms may continue development until the intracellular concentration becomes high. With the much higher concentrations of the reagents, about 0.1 per cent and in some cases even one per cent, in some of the earlier work with the reaction and with alkali added to dissolve the α-naphthol, the material must have been killed almost at once, and little or no gradient pattern remained. For the echinoderm material twice the standard was commonly used. somewhat less often, the standard solution.

Some organisms apparently reduce or otherwise destroy intracellular indophenol with partial or complete loss of color, after the reaction has continued for a time, perhaps because of stimulation or irritation by the intracellular indophenol or the aniline. This decrease or loss of color has sometimes been observed in later echinoid blastulae and in gastrulae. Also, unless the intracellular concentration is very high there is usually more or less reduction with loss of color when cytolysis occurs. Intracellular reduction occurs rapidly on addition of very small amounts of sodium hydrosulphite, provided the intracellular concentration of indophenol has not become so high that reduction is retarded or inhibited. Re-oxidation and perhaps a second reduction are often possible without alteration of pattern.

The patterns of primary intracellular oxidation from dye solutions reduced by sodium hydrosulphite and of indophenol are similar but the indophenol reaction seems to show very slight differentials in the echinoderm material a little more distinctly than dye oxidation. Reduction patterns of the dyes and of indophenol are also similar. It is perhaps unnecessary to point out that the earlier stages of the indophenol reaction and also of primary dye oxidation are more important than the later stages in making visible the regional differentials of pattern. With increasing intracellular concentrations of oxidized dyes and of indophenol, slight differentials become less distinct or disappear as the color approaches uniformity. With low concentrations of dyes and indophenol reagents these methods are an exceedingly delicate means for showing directly slight regional differentials in activity of an oxidase or of oxidases and in reduction, of one or more dehydrogenases. They have an advantage over various other methods of not requiring separation of the organism into pieces.

In observation of these indicator patterns the most extreme precautions have been taken to avoid being deceived by apparent differentials resulting from direction or character of illumination or other extraneous factors. Frequent agitation has provided for uniform concentrations of dyes and indophenol reagents in non-motile stages. In motile stages the swimming activity of the animals serves this purpose. Single eggs have been moved about and turned over repeatedly. The patterns noted in this paper have been seen in hundreds of individuals during earlier and

later parts of the breeding seasons and in different lots of material during the last five years.

Figures are diagrammatic outlines with the gradients indicated by differential shading or by arrows pointing in the general direction of decrease in rate of intracellular oxidation, *i.e.*, down the gradient. Early cleavages of *S. purpuratus* and of *Dendraster excentricus* are alike. *Dendraster* eggs and cleavage stages are larger than those of the sea urchin but separate figures for these stages of the two species are regarded as unnecessary. The egg and developmental stages of the starfish, *Patiria*, are much larger than those of the echinoids and are outlined in the figures as slightly larger, but do not indicate the actual differences in size. In the shaded figures the shading is an attempt to indicate the differential as observed in particular cells, and nuclear position in the cell is drawn as observed. Arrows indicate merely the general directions of gradients, not their extent. Shorter arrows indicate less differential.

Growth Stages of Ovarian Oöcytes

In the earlier study of intracellular reduction of redox dyes in the ovarian oöcytes of *S. purpuratus*, *S. franciscanus* and *Patiria miniata*, rate of reduction usually decreased from that region of the cell where the nucleus was nearest the surface (Child, 1936a; Figs. 1–3, 8 and 9). This region varied with respect to attachment to ovarian tissues but was usually somewhere on the surface not in contact with ovarian tissues, though a few very early oöcytes were observed with reduction gradient decreasing from the attached pole and nucleus near that pole (Child, 1936a; Fig. 4). These may have been stages so early in oöcyte growth that other ovarian tissues still reduced more rapidly than the oöcyte. Figure 5 of the same paper with reduction decreasing in rate from the interior of the cell after staining by oxidized diazine green, is probably a case of differential injury with retardation of reduction near the cell surface; with intracellular oxidation nothing similar has ever been observed.

It is usually possible to find early oöcyte stages by gentle teasing of small portions of the ovaries, even in females with large numbers of eggs ready for fertilization. Figure 1, *a–x*, the result of repeated series of observations on all three echinoderms constituting the material of this paper, is believed to be a fairly adequate sample of the gradient patterns observed in the earlier stages of oöcyte growth and of the nuclear positions in relation to these patterns. In Figure 1, *a–l* are from *S. purpuratus*, *m–q* from *Dendraster* and *r–x* from *Patiria*. In most of these cells the relation to the ovarian tissue is not evident. In those which do show some indication of this relation (Fig. 1, *f*, *k*, *n*, *o*) the region of most rapid intracellular oxidation is at or near the "free" pole of the cell. The oöcytes differ in form and are often more or less elongated, the axis of elongation being the gradient axis or departing only slightly from it. The gradient pattern in these cells is distinct, though with apparent variation in differential in different cells. In most cases the rate of oxidation decreases rapidly from one pole with relatively slight differential in the remainder, often a half of the cell. This condition has seemed to be more distinct in *S. purpuratus* and *Dendraster* than in *Patiria*, but whether it is of real significance remains uncertain. It is apparently more frequent in the earlier stages and may perhaps indicate that the gradient is in process of developing from the high end.

Nuclear position in relation to the gradient pattern varies. In the earliest stages (Fig. 1, *a–d, r*) it may be at or near the pole of least rapid reaction, the "low" end of the gradient or in some other region of the cell, perhaps near the middle (*g, j, k, s, w*) and not always in the gradient axis. However, in the great majority of the oöcytes the nucleus is near the high end of the oxidation gradient and apparently more frequently in this position as size of the oöcyte increases, but a cell approaching full size has occasionally been seen with nucleus near the middle. These observed differences may be without real significance, but if they do represent actual physiological differences in individual cells they suggest that gradient pattern is developing in the oöcytes, that different stages of this development appear in different cells and that the nucleus gradually comes to lie near the high end of the gradient, though some variation in position may still occur in later stages.

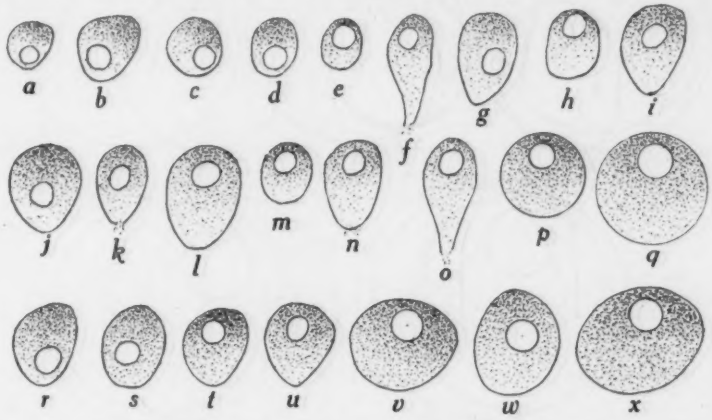

FIGURE 1. Gradient patterns of early oöcytes: *a–l, S. purpuratus; m–q, Dendraster excentricus; r–x, Patiria miniata.*

As the cell increases in size the gradient differential seems to become less distinct, *i.e.*, the lower gradient levels differ less from the higher levels than in many earlier stages, and in general the gradient appears to decrease in rate of reaction, probably as enzyme activity decreases in the later stages of growth. However, it is difficult to determine whether changes such as this are or are not significant. It is certain that the oxidation gradient pattern does become less distinct in the full grown unfertilized egg than in the early oöcyte stages.

All oöcytes of Figure 1 are placed in the figure with the region of most rapid oxidation uppermost. This involves the assumption that the gradient pattern represents a definite physiological axis, rather than a mere chance differential differing from cell to cell and without further significance. Moreover, even though these early stages alone do not provide evidence that the high ends of their gradient patterns become the apical or animal pole of the egg, comparison with later stages leaves little doubt that this is the case. It appears highly improbable that a gradient pattern involving enzymes of fundamental importance is without definite relation to oxidation patterns of later stages. It is believed, therefore, that these data con-

cerning early stages in ovarian egg development indicate a developing apicobasal physiological axis that persists and becomes the polarity of the egg and embryo.

With further growth of the oöcyte the relation of nuclear position to the high end of the indicator gradient becomes increasingly definite, though some variation still occurs. The usual nuclear position in later growth stages is indicated in Figure 2, but even in these stages an oöcyte with nucleus near the center of the cell has occasionally been seen. These cells are perhaps not in good condition. With progress of growth the indophenol reaction becomes much less rapid and the gradient differential is slight but still distinguishable with care in use of the reaction and in observation. As it approaches the end of the growth period the cell evidently becomes less active, at least so far as the oxidase or oxidases catalyzing the

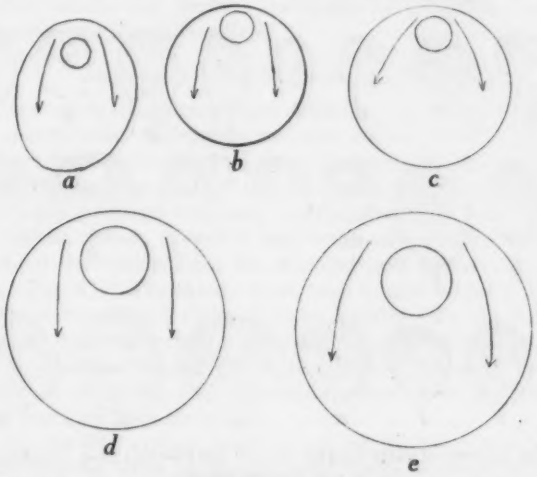

FIGURE 2. Gradient patterns of later growth stages of oöcytes: *a–d, S. purpuratus* and *Dendraster; e, Patiria.*

reaction are concerned, and preceding fertilization, or, in the case of the starfish, preceding polar body formation, it is an extremely inactive cell. In many of these cells, however, a slight gradient is still visible; in some others it has not been distinguished with certainty.

POLAR BODY STAGES

In the two echinoids the first polar body is formed in the ovary preceding spawning. In slightly teased ovaries it has been seen occasionally (Fig. 3, *a*, *b*). It is formed at the high end of the slight gradient. It has sometimes seemed that the gradient became slightly more distinct at the time of its formation. There is every reason to believe that the pole near which the nucleus lies in Figure 2 and the region of polar body formation are the same. It appears highly improbable that the nucleus has moved to another region of the egg after the growth period of

the oöcyte has ended. The nucleus of course becomes invisible at the time of polar body formation and afterward it is smaller but can usually be made visible by pressure (Child, 1936a; Fig. 7). The second polar bodies of the two echinoids are formed after fertilization (Fig. 3, *c, d*). The intracellular indophenol reaction becomes more rapid and the gradient more distinct following fertilization. The *Patiria* eggs were fertilized as soon as possible after extrusion from the gonads and both polar bodies formed after fertilization (Fig. 3, *e*), also with increase in distinctness of the indophenol gradient. If the *Patiria* eggs had been kept without

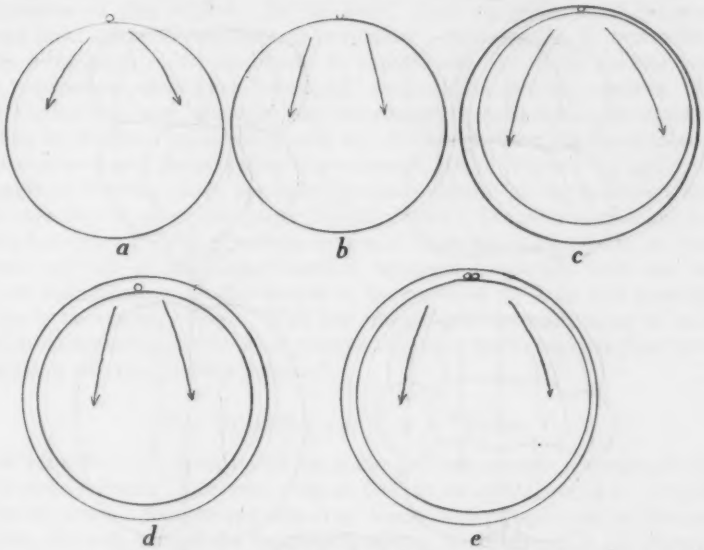

FIGURE 3. Gradient patterns of polar body stages: *a* and *b*, first polar body stage of *S. purpuratus* and *Dendraster* before fertilization; *c* and *d*, second polar body stage of these forms following fertilization; *e*, polar bodies of *Patiria*, following fertilization.

fertilization, polar body formation and activation would probably have occurred as in *Asterias* (Loeb and Wasteneys, 1912; Tang, 1931). Polar bodies formed at the pole of most rapid indophenol reaction.

EARLY CLEAVAGE STAGES

The gradient pattern in early cleavage stages of *S. purpuratus* has been rendered visible by primary intracellular oxidation of reduced methylene blue, diazine green and Nile blue sulphate as well as by the indophenol reaction, the same pattern appearing with all three procedures. Only the indophenol reaction has been used on most lots of *Dendraster* and *Patiria* with the same results in all cases. The observations on these stages have been made repeatedly on many lots of eggs during different breeding seasons from 1947 on. They have included thousands

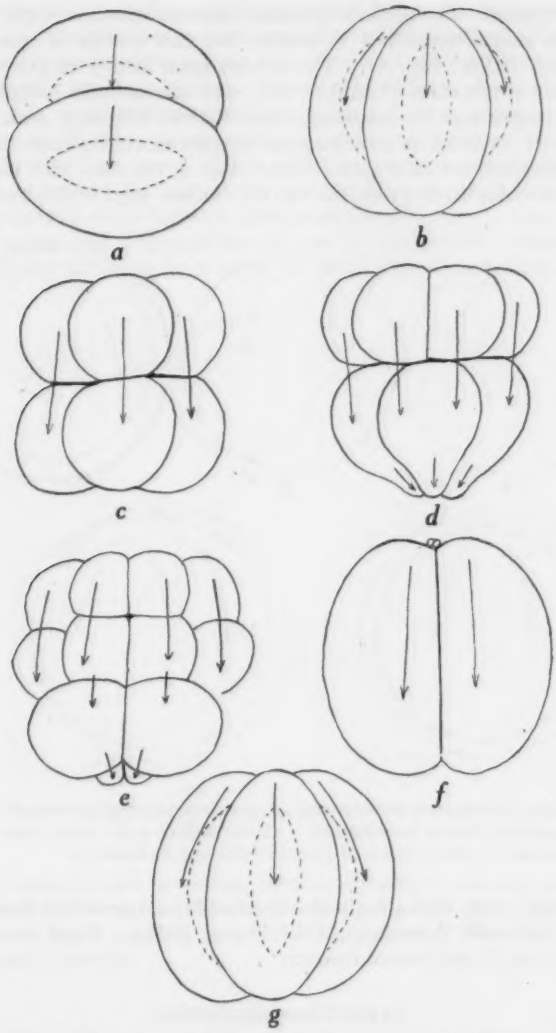

FIGURE 4. Gradient patterns of early cleavage stages: *a* and *b*, 2- and 4-cell stages of *Strongylocentrotus* and *Dendraster* with outlines of mitotic figures for next division; *c* and *d*, 8- and 16-cell stages of the two echinoids, difference between mesomeres and macromeres distinct, between macromeres and forming micromeres less distinct but visible; *e*, 24-cell stage of the echinoids, difference between mesomeres and macromeres apparently slightly more distinct in many embryos, in others essentially like 16-cell stage, micromeres still slower reaction than macromeres; *f* and *g*, 2- and 4-cell stages of *Patiria*.

of eggs under widely varied conditions of illumination. Early cleavages of *S. purpuratus* and *Dendraster* are alike, and in Figure 4, *a–e* indicate gradient pattern in both. In these forms the second polar body usually lies deep in the cleavage furrow and is often not certainly visible. For certain orientation, in Figure 4, *a*, the 2-cell stage is drawn with outlines of the mitotic figures for the second cleavage indicated. In Figure 4, *b*, the 4-cell stage, mitotic figure outlines for the third cleavage are also indicated. These are slightly nearer the pole of most rapid dye oxidation and indophenol reaction and the four apical cells of the 8-cell stage are usually slightly smaller than the basal cells (Fig. 4, *c*), though there is apparently some variation in this respect. In the 8-cell stage the differential between the apical and basal cells becomes even more distinct. In Figure 4, *d*, approaching 16 cells, the differential from mesomeres to macromeres is clearly evident and the forming micromeres show even less rapid reaction than the macromeres. At the stage of Figure 4, *d*, dye oxidation and indophenol reaction are more rapid in the apical than in the basal mesomeres with less difference from the basal mesomeres to the macromeres and the micromeres are slowest of all. Figure 4, *f* and *g*, 2- and 4-cell stages of *Patiria*, shows the same gradient pattern as the echinoids and the same as observed in an earlier paper (Child, 1944). No micromeres are formed in *Patiria* but there is a slight increase in size of blastomeres basipetally as cleavage progresses and rate of indophenol reaction decreases basipetally from the smaller cells of the apical region, as also shown in the paper of 1944, so that repetition of those data is unnecessary here. With low intracellular concentrations of oxidized dyes and indophenol it has often been possible to reduce and re-oxidize these cleavage stages without altering gradient pattern.

The Blastula and Later Stages

In the blastula of all three forms the polar gradient pattern becomes so distinct that it is clearly visible in surface view as well as in optical section. A point of interest to be noted is that the reaction at all levels is more rapid on the blastocoelar side of the cell wall, not on the external surface. In Figure 5, *a* (*S. purpuratus* and *Dendraster*) and *b*, *Patiria*, the arrows in the cell wall indicate the gradient in optical section, the other arrows the gradient in surface view. The differential from the blastocoelar surface outward is indicated only at the apical end by the optical section arrows drawn from the blastocoelar surface. Thus far it has not been possible to distinguish with certainty a ventrodorsal gradient in blastulae. In some blastulae intracellular oxidation seemed to be slightly more rapid on one side but even if this is actually the case it is not certain that the more rapid side is ventral, as it is in later stages, or whether any probable differential is due to some incidental factor. Probably a ventrodorsal pattern is present at this stage and even earlier but it is either too slight to become distinctly visible in intracellular oxidation or may conceivably differ in character from the ventrodorsal gradient of later stages. In Figure 5, *c*, the gastrula of *S. purpuratus* and in *d* the gastrula of *Dendraster* with somewhat longer apicobasal axis are outlined. Polar and ventrodorsal gradients are both clearly visible in the ectoderm. In the invaginating entoderm a new longitudinal gradient has arisen with rate of oxidation decreasing from the tip. In Figure 5, *e*, the pre-pluteus or early pluteus of the echinoids is outlined in somewhat oblique optical section from the side, with the gradient pattern indicated. The

ectodermal polar gradient is becoming more distinct as the oral lobe develops from the apical region. The ventrodorsal gradient is also more distinct than in earlier stages, and a new gradient decreasing from the tip of each developing anal arm is now present. In stages *c*, *d* and *e* the ectodermal pattern is sufficiently distinct to be visible in surface view and optical section. The entodermal differential has also increased. As regards the intracellular indophenol pattern of the later stages of *Patiria* it is unnecessary to repeat here the data recorded in the earlier paper (Child, 1944).

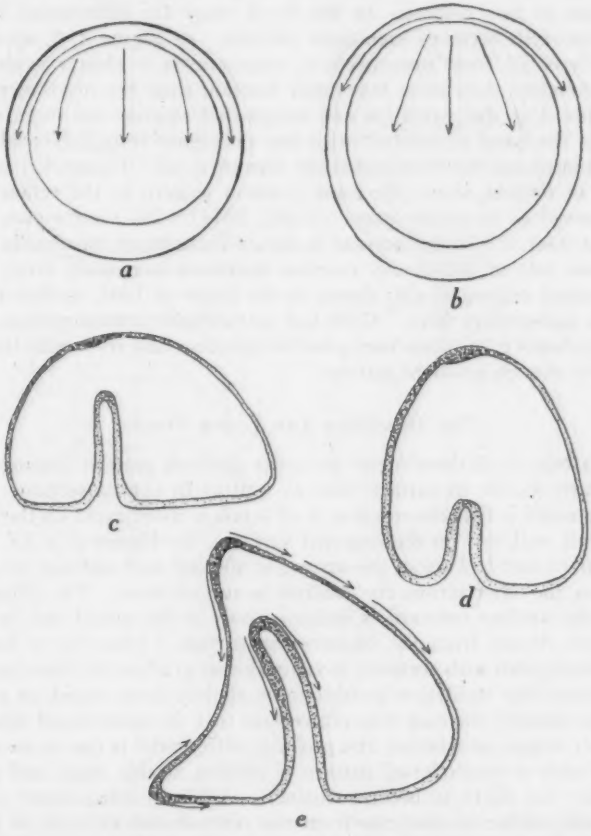

FIGURE 5. Gradient patterns of later stages; differential becomes increasingly distinct during blastula stage. Arrows in cell wall indicate gradient in optical section, other arrows, gradient in surface view. *a*, the two echinoids; *b*, *Patiria*; *c*, gastrula of *S. purpuratus*; *d*, gastrula of *Dendraster*, apicobasal, ventrodorsal and entodermal gradients indicated in both; *e*, somewhat oblique outline from side of early pluteus of echinoids, gradient of developing oral lobe in apical region, ventrodorsal gradient, gradient in developing anal arm and gradient in entoderm indicated.

Without feeding, the echinoid plutei gradually die of starvation. During the later stages of their lives intracellular oxidation of reduced dyes and of indophenol reagents becomes less rapid and gradient differentials decrease. Even while they are still to some degree motile they show little evidence of oxidase activity and little or no gradient pattern. Starving larvae of *Patiria* show very similar decrease in gradient pattern and of oxidase activity.

Discussion

An intracellular oxidation gradient pattern of indophenol reagents and of various reduced redox dyes is present through ovarian development of the oöcyte and through embryonic and larval development. Intracellular oxidation is more effective than reduction in rendering visible slight differentials; staining by oxidized dyes preceding reduction may itself decrease differentials. The differential developmental modifications by external agents show the very definite relations of morphogenesis to this gradient pattern. In the early oöcytes the gradient pattern is very distinct but becomes less so as developmental activity of the oöcyte decreases in later stages. The course of oöcyte development suggests that the cell acquires a cytoplasmic gradient pattern from a differential in ovarian environment, perhaps an oxygen differential, and that the nucleus gradually attains a position near the most active region of this gradient, polar bodies form there and the gradient becomes the basis of physiological polarity in the embryo and larva.

A ventrodorsal pattern of some sort is probably present long before it becomes distinguishable by means of the indicators. It is perhaps of interest to note that in the growth of the ovaries the small finger-like outgrowths show an indicator oxidation gradient decreasing from their tips. Perhaps the oöcyte is subjected from its earliest stages to an ovarian differential at right angles to the polar differential. If this determines a differential in the cell it may be either too slight in early stages to appear, even in intracellular oxidation of indicators, or may not yet have developed a differential in oxidase activity.

The present paper makes it necessary to refer again to the failure of Lindahl and Holter (1940) to find any significant difference in oxygen uptake in apical (animal) and basal (vegetal) halves of developmental stages of *Paracentrotus lividus* and to the somewhat later assertion of Lindahl (1942a, 1942b) that the data of Child concerning reduction gradients in a sea urchin cannot be "valid" because no differential in oxygen uptake was found in the separated halves of embryos. This matter was discussed in an earlier paper (Child, 1944) and only certain points need be noted here. Lindahl's assertion concerning validity seems not entirely justified on the basis of his and Holter's purely negative evidence and in the face of positive evidence to the contrary. These authors do not mention any attempt to determine whether a much larger number of embryo halves than they used would show a differential. Also an error in one of their tables invalidates certain of their conclusions. Moreover, they admit an error of 10 per cent. It has never been maintained that indicator gradients must always parallel regional differentials in oxygen uptake, though they are parallel in various organisms. It is conceivable that separation of the echinoderm stages into parts may decrease or obscure regional differences in oxygen uptake, particularly if these are slight. Possibly also other factors may sometimes be involved in oxygen uptake than those determining indi-

cator patterns. The indicator methods have certain advantages over even Cartesian diver methods of determining oxygen uptake. Although they are not quantitative they are more delicate than any other method and make directly visible very slight regional differentials even in very small cells without isolation of parts. There can be no question concerning validity of the data of the present paper. With care in use of indicators and in observation, these gradient patterns can be seen by anyone not color blind and with fairly good visual perception of slight differences in depth of color. Also, with a little practice ability to distinguish slight differentials with a high degree of certainty increases.

The critic may question whether the observed gradient patterns are actually the same in different oöcytes and at different stages or whether they are merely chance differentials. In those early oöcytes showing evidence of relation to ovarian tissues the most active ends of the gradients are at or near the opposite pole. The increasingly definite relation of the nuclei to these more active gradient regions, the formation of polar bodies there and the relation of gradient patterns to physiological axes indicate their significance. In this connection it is of interest that, according to Tennent (1931), the first polar body of the sea urchin, *Mespilia globulus,* forms at the free pole of the ovarian cell.

Since this paper is concerned with gradient patterns, the occasion is taken to refer to the interesting experiments on alteration of ventrodorsality by subjection of *Dendraster* embryos at the 8-cell stage to a concentration gradient of various agents (Pease, 1941, 1942a, 1942b). Some of the agents used are known or believed to inhibit certain enzyme systems, with perhaps still other effects; actions of some others are perhaps less well known. The effective agents determined the most inhibited region as dorsal and the less inhibited became ventral with a frequency sufficient to demonstrate a positive determining action. Indicators do not show ventrodorsality in early embryonic stages, though it is doubtless present in some degree or form (see also Hörstadius and Wolsky, 1936). When it becomes visible as an indicator gradient it decreases from the ventral side dorsipetally. There is no distinguishable difference between right and left sides in early stages. Pease speaks repeatedly of his determination of bilaterality, although he actually determines ventrodorsality. Since the embryo has three spatial dimensions and since there is no evidence of asymmetry in the stages concerned, bilaterality is only an incidental consequence of determination of ventrodorsality.

In use of various agents Pease found that inhibition of cleavage and determination of ventrodorsality were not necessarily associated in action of a particular agent. To quote him concerning this point: "This leads to the conclusion that the bilateral (sic) determination is not dependent on a vague metabolic gradient but is rather dependent upon a specific enzyme system or linked systems" (Pease, 1941; p. 399). And again: "Bilateral (sic) determination by chemical concentration gradients is not dependent upon general metabolic gradients because this determination can be separated from differential cleavage inhibition" (Pease, 1942b; p. 352). The meaning of these statements is not clear. Do they mean that the ventrodorsality which he has determined is different in physiological character from the ventrodorsality of the uninhibited embryo? If this is the meaning, two different sorts of ventrodorsality can result in the same course of ventrodorsal development. This appears highly improbable. Moreover, cleavage, though to some degree correlated with gradient pattern, also unquestionably involves various activities which have

little or no relation to that pattern. Some agents may inhibit these without any effect on ventrodorsality. There is every reason to believe that the experimentally determined ventrodorsality is the same as ventrodorsality in uninhibited development. Examination and counts of the material were made at the late gastrula or "prism" stage, but the question whether the experimental ventrodorsality is so completely determined that the fully developed pluteus form is attained or whether there is some differential inhibition, is not considered by Pease.

Pease also says: "The extreme sensitivity of the bilateral (!) determination to concentration gradients suggested that respiratory mechanisms might be involved (Pease, 1941). The further successful experiments with azide demonstrated with little probability of error that an oxidation system passing through a heavy metal catalyst is important in this determination" (Pease, 1942, pp. 352–3). The following pages of the summary of his paper are largely concerned with hypotheses concerning the enzyme systems involved in the determination of ventrodorsality, He seems not to be aware that according to present conceptions the action of redox indicators depends on an oxidase or oxidases and on one or more dehydrogenases. Moreover, various agents effective in determining differential developmental modifications are known to be oxidase or dehydrogenase inhibitors, or more or less general inhibitors of enzyme activity. If these respiratory enzyme systems are as important in the ventrodorsal determination as Pease maintains, there can be no doubt that the experimentally determined ventrodorsal gradient is the same physiologically as the gradient made visible by the redox indicators in uninhibited development. Incidentally, although Pease is evidently convinced that he has determined bilaterality, he does not consider how the external gradient could determine a bilaterality at a right angle to its own differential and without distinguishable difference on the two sides.

Pease's experiments suggest that a certain degree of physiological dominance of the ventral region, the high end of the ventrodorsal gradient pattern according to the redox indicators, over the less active dorsal region is present in unaltered development. With inhibition of the dominant ventral region, the dorsal region becomes more or less physiologically isolated, its activity increases and it develops as a ventral region. The experiments suggest further that in early developmental stages the difference between ventral and dorsal is at least very largely, if not entirely, quantitative.

The indicator gradients and the differential developmental modifications also suggest that in the early development of the echinoderms and of many other organisms regional differences in at least certain enzyme systems are predominantly or entirely quantitative, and that regionally specific differences gradually arise. Does not differentiation consist largely in regional localization of certain enzyme systems, while certain others remain less definitely localized?. Apparently oxidases and dehydrogenases, or certain of them, are among those which usually remain rather generally distributed. In many organisms quantitative differentials in indicator patterns persist throughout life.

Most of our knowledge, as distinguished from hypotheses concerning enzymes, has been obtained by destruction of living protoplasms. We know little concerning the internal relations of enzyme systems in living, apparently uninjured organisms. The indicator methods are not quantitative but they give information which at present can be obtained in no other way. Also the differential developmental

modifications give information concerning the significance of the indicator gradient patterns in developmental morphogenesis, and it appears probable that with further progress and refinement in use of external agents, they will give information concerning other than the respiratory enzyme systems.

In view of the present interest in enzyme systems it seems desirable to call attention to another aspect of action of an external agent on living organisms. In a system in which continuous correlated change is occurring, the rate of that change, in general terms the activity of the system, may be an important factor in determining its sensitivity or susceptibility to an external agent. Insofar as quantitative differences in activity are characteristic of the system or its parts, susceptibility to an external agent which retards, inhibits or otherwise alters any essential quantitative factor of the system, and so alters the system, will vary with the activity. The effect of the agent will occur more rapidly and in greater degree in the more active than in the less active system or part, irrespective of the particular character of the action of the agent. In the echinoderm eggs and embryos regional quantitative differentials in at least certain of the correlated changes characteristic of the gradient pattern are present, though not necessarily the only regional differences present. Susceptibility to any agent which acts on an essential factor of this quantitative differential will be greater in the more active than in the less active gradient levels. In other words, the action of the agent will be differential in relation to the gradient pattern; by an inhibiting agent the more active "higher" gradient levels will be more inhibited than less active levels, whatever the particular factor on which the agent acts. Also, recovery from temporary action will be more rapid and more complete at more active than at less active levels, provided action of the agent has not become irreversible, and equilibration or development of tolerance and acclimation will show the same relation to the gradient pattern. This relation of susceptibility to external agents and activity accounts for the determination of similar differential developmental modifications by many external agents which certainly do not all act in the same way on a protoplasm. And conversely, these similar modifications by different agents constitute evidence that in the echinoderms and various other organisms quantitative differentials are important factors in gradient pattern. This relation of susceptibility to activity by no means excludes the possibility that certain agents may provide evidence of specific regional differences in effect; perhaps some modifications of echinoderm development are suggestive of such effects.

The relation of susceptibility to activity holds for inorganic systems with quantitative differences in activity as well as for living organisms. For example, a rapid stream or a rapidly moving automobile is more susceptible to a sufficient degree of disturbance of any kind than a slow stream or car, and recovery from less extreme temporary disturbance and equilibration to a slight disturbance show the same relation to activity.

In conclusion, on the basis of evidence at present available it appears beyond question that a gradient pattern, primarily predominantly or entirely quantitative, is an essential factor in echinoderm larval development. Moreover, this pattern provides a physiological basis for activation of different genes in different cells or cell groups, *i.e.*, for differentiation. When this pattern is altered experimentally the course of morphogenesis is altered; when gradient pattern is obliterated, development and differentiation do not occur unless a new gradient is determined by an external agent, as has been done in certain organisms.

SUMMARY

1. With progress in the use of redox indicators, it has been found that slight gradient differentials become more distinctly visible by intracellular oxidation of redox dyes, reduced by essentially non-toxic sodium hydrosulphite, and of low concentrations of indophenol reagents than in reduction of oxidized dyes. Staining by oxidized dyes preceding reduction may itself decrease slight differentials.

2. By means of intracellular oxidation of reduced dyes and indophenol reagents a gradient pattern has been rendered directly visible from early oöcyte to larval stages. The evidence indicates that the gradient of the early oöcyte becomes the polar gradient of the egg and embryo. In early oöcyte stages, position of the nucleus varies, but as oöcyte growth progresses it comes to lie near the pole of most rapid intracellular oxidation, the polar bodies form there and this becomes the apical (animal) pole of egg and embryo.

3. The ventrodorsal gradient pattern becomes visible in the gastrula, perhaps in the late blastula, but is undoubtedly present earlier, probably with a differential too slight to be visible or in another physiological condition. Other gradients appear in the further course of development.

4. Experiments of Pease on determination of ventrodorsality in *Dendraster* by gradients of inhibitory agents are discussed in relation to redox gradient pattern.

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EFFECTS OF CHEMICALS ON A SCHOOLING FISH, KUHLIA SANDVICENSIS [1, 2]

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Recent widespread interest in the reactions of fish and other aquatic vertebrates to chemical substances has prompted us to place on record certain significant results which have accrued during the preliminary phases of an investigation directed toward dispersing schooling fish by chemical stimuli. This study was undertaken to find, by judicious selection and testing, whether or not certain chemical compounds in exceedingly dilute concentrations would evoke reactions in fish which would alter the sensory bonds between them, thus effecting the desired dissolution of the school. Although much additional research is yet to be accomplished on this problem, the results thus far point toward promising leads and will, we anticipate, be of considerable value to other workers engaged in closely related lines of investigation.

A chemo-sensory approach to the problem of dispersing fish in schools appears justified on the basis of (1) the role of vision in the formation, maintenance, and dissolution of fish schools as demonstrated by Parr (1927, 1931), Breder (1929, 1942, 1951), Bowen (1931, 1932), Breder and Nigrelli (1935), Johnson (1939), Schlaifer (1940) and others, and (2) the chemical sensitivity of fish as indicated by the early studies of Parker (1912), and a host of others up to the present day. It is clear in regard to vision that whatever may be the total forces which weld and maintain the fish school, dissolution of the school occurs when light intensity falls below a certain threshold value. Moreover, many experiments with blinded fish of species which school under normal conditions indicate that schooling fails to occur under these altered circumstances. For these reasons one of the specific objectives of this study is to discover chemical compounds capable of inducing amblyopia. As regards the chemical sensitivity of fish a search is underway to segregate from promising chemicals those which evoke the irritating or repelling responses desired. Upon this background of information further tests are being conducted with (1) closely related molecules with different substituents and (2) structurally analogous compounds. Finally, we hope to combine active parts of molecules into substances which may increase the intensity of response.

A survey of the literature indicates that the reaction of fish to chemical substances has not been a fertile field of investigation except insofar as certain specific piscicides, insecticides and pollutants are concerned. The history, use and effectiveness of piscicides have been reviewed recently by Krumholz (1948), Solman (1950) and Smith (1950). These authors stress rotenone as the most effective fish poison

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for use in fisheries work. Hubbs (1930) found nascent oxygen to be extremely toxic to fish, and Behrens and Hikiji (1933) discovered that central respiratory stimulants (cardiazole, camphor, picrotoxin, lobeline and rotenone) were generally toxic to fish although there is no strict structural parallelism. Throughout recorded history certain plants have been known to contain substances toxic to fish. Some of these are discussed by Fickendey (1911), Chopra (1941) and Bianco (1943). Very scant information exists on the active compounds or mode of action on fish of any of these piscicides.

Many of the newer insecticides and weed killers which are applied over extensive areas have posed problems in fish conservation. The toxicity of DDT to fish has been discussed by numerous workers, among them Ginsberg (1945, 1948) and Linduska and Surber (1948). Dilute concentrations of pyrethrum (Bandt, 1933), phenol larvicides (Knowles, Parker and Johnson, 1941), and 2,4-D (Harrison and Rees, 1946) have also been found destructive to fish.

The reaction of fish to chemicals as mediated through the olfactory sense has received considerable attention particularly since the experimental results obtained by Parker (1911, 1913), Parker and Sheldon (1914) and others who differentiated general chemical sensibility in fish into the separate receptors, stimuli and physiological effects of smell, taste and the common chemical sense. Recent studies by Hasler and Wisby (1949) have demonstrated that conditioned fish can detect phenol at concentrations of 0.0005 p.p.m. That odoriferous substances may evoke an alarm response has been demonstrated by Von Frisch (1941) who proved that the injured skin of the European minnow, *Phoxinus laevis*, gave off a substance, perceived through the olfactory epithelium, which initiated a fright response among its "school mates." Hüttel (1941) found this substance to be purine- or pterine-like. Unfortunately no quantitative data are available for comparatively assessing the response. Because of the extreme sensitivity of fish to odors, stimuli mediated through the olfactory sensations may well play an important role in the life of fish, serving to guide them to feeding grounds, to direct migrating fish to their homing areas, and to warn fish about toxic substances present in the environment (Walker and Hasler, 1949). In regard to the latter point responses of fish to pollutants have provided a considerable amount of the literature relating reactions of fish to chemical substances, especially those perceived through olfaction. The effect of phenolic substances on fish has been reviewed and extended recently by Jones (1951), toxicity levels for fish in waters polluted with hydrocyanic acid and other industrial by-products have been examined by Daugherty and Garrett (1951), and fatal concentrations of zinc for trout have been recently determined by Goodman (1951). Such responses by fish to pollutants have indicated their usefulness in the bioassay of chemical by-products (Hart, Doudoroff and Greenbank, 1945; Hasler and Wisby, 1949; Daugherty, 1951).

It is abundantly clear from the literature that the three chemical senses as described for fish by Parker (1912) are susceptible to different types of stimuli and that there is great disparity in thresholds for stimulation, especially between olfactory sensation and those of taste and the common chemical sense. Although there is no comparative information available for fish on this disparity in threshold of stimulation, tests have been conducted with humans which provide much data germane to our investigation. Katz and Talbert (1930) measured the intensities

of stimulation of 55 different substances for odor, eye irritation and nasal irritation in human subjects. They found that trinitro tertiary butylxylene (artificial musk) was the most powerful as an odor and was observable at 0.00005 p.p.m., while phenacyl chloride was the most powerful eye irritant, observable at 0.0083 p.p.m., and was also the most powerful nasal irritant, discernible at 0.021 p.p.m. It is interesting to note that the irritant receptors of the human eye are two or three times as sensitive as those in the mucous membranes in the nose, but are nevertheless 160 times less sensitive than the olfactory apparatus. At this stage in the development of a comparative physiology of the chemical senses we are not permitted to expect fish to respond to stimuli at the same threshold levels as do humans; nonetheless, it may be instructive to deduce possible proportionate threshold intensities on the part of fish from the meager data available. The maximum detectable dilution of a chemical substance in fish mediated through the olfactory receptors appears to be approximately 0.0005 p.p.m. (Hasler and Wisby, 1949). Thus, if the proportionate threshold intensities of minimal stimuli for the general chemical senses of humans and fish are somewhat comparable, we would expect that the eyes of fish might possibly be irritated by a suitable compound in a concentration as dilute as 0.08 p.p.m., and that the common chemical sensory receptors in the skin might be irritated similarly by a concentration as dilute as 0.3 or 0.4 p.p.m. These estimates provide us with a point of departure and a frame of reference for the quantitative aspects of this study.

Too little information is available to point definitely to any of the chemical senses as demanding our sole attention toward the solution of the problem. The greater sensitivity of olfactory receptors holds the possibility that certain chemicals in very dilute amounts might induce the desired response. However, none of the responses attributable to such chemicals appears to evoke behavior which would disperse schooling fish. In general, prior studies on the physiology of taste in fish have indicated that these stimuli evoke positive reactions to food, whereas negative or defensive reactions are mediated through the common chemical sense. Recent anatomical and physiological studies of the fishes *Prionotus* and *Trichogaster* indicate that the difference between the common chemical sense and taste can only be based on the innervation and the presence or absence of taste buds (Scharrer, Smith and Palay, 1947). Thus, the reaction of the animal cannot safely be used as a criterion for precise differentiation of stimulus-response patterns for these two senses. Nonetheless, the sensations resulting from stimulation of the receptors of the common chemical sense are more closely allied to the sense of pain (Moncrieff, 1944) and thus should provide the desired behavior. Since vigorous and defensive actions are the types sought in this study, the bulk of our tests involve chemicals eliciting this type of response which may be generally considered to be mediated through the free nerve endings. These, although we are not certain for this species, are presumably located over the surface of the body.

Very little information is available concerning repellent action of chemicals on bony fish. While conducting experiments on Mexican blind characins Breder and Rasquin (1943) found that acetic acid, 10 per cent, and ammonium carbonate were repellent. At the onset of World War II extensive investigations were conducted to develop a suitable shark repellent, and cupric acetate as well as other acetates was found fairly effective (Burden, 1945; McBride and Schmidt, 1943). Further

analysis indicated that acetic acid was the active repelling agent when these substances were dissolved in water, but no suitable volumetric information is available. Since the War the same material was tested and found effective for sharks and several species of bony fish in Australian waters (Whitley and Payne, 1947).

Selection of Chemicals

The basic considerations for selecting chemicals capable of dispersing schools of fish were (1) that they be grossly irritating to fish rather than merely toxic or narcotic, and (2) that they be rapid in action, reaching, in our experiments, a maximum effectiveness within a period of two or three minutes. In searching for suitable chemicals we were aware that substances which would elicit these reactions in other groups of animals might not be effective likewise for fishes. However, as a point of departure it was assumed that reactions to chemicals by other well-studied groups of animals would be helpful. Accordingly, the kinds of information which led to the initial choice of chemicals for testing were (1) actions of insecticides and insect repellents, (2) general knowledge of mammalian toxicants, stenches, irritants and lachrymators, and (3) previous results with chemical piscicides and fish repellents. After preliminary tests with substances in these categories it soon became evident that most of the desired chemical properties were obtainable in the group of irritant poisons classified usually as lachrymators and skin irritants. These substances probably impart their effect to fish through receptors of the common chemical sense as conceived by Parker. The selection of test chemicals was thus further narrowed to the classes of substances known to possess these properties from experimental results obtained for other animals, chiefly insects and mammals. Lest this system of selection miss chemicals or classes of chemicals which might be irritating to fish, but not to other animals thus far studied, other chemicals considered possibly effective have been included in the testing program.

The property of irritation is generally accorded to a chemical by the presence of characteristic groups in the molecular structure. However, an attempt to classify known irritants according to such active groups reveals many significant exceptions, so that *a priori* such a scheme does not appear very promising. Nonetheless, the many relations between structure and irritant power seem to demonstrate the anlage of a skeleton on which a classification will almost certainly be built. Other factors, such as those which control the amount of irritant reaching the sensory receptors (vapor pressure in air, and solubility in water), are certainly important contributors to the efficacy of stimulation. Upon these qualifications presumably active chemicals of the following groups have been selected for the testing program: mercaptans, sulfides, thiocyanates, isocyanates, isothiocyanates, phenols, highly halogenated compounds, and certain classes of unsaturated compounds. Those with lower molecular weight were also given priority in testing because of the greater solubility expected.

Classification of chemicals tested

A preliminary classification of the chemicals tested thus far follows. It will be noted that certain of the substances listed have properties which fit them into more than one category, and thus make them doubly recommended for testing.

a. *Insecticides and insect repellents*

Several insecticides and insect repellents have been tested. With regard to their stimulation of the common chemical sense in man these substances would fit into no single classification, but would run the gamut from bland to mildly irritating. We have explored the action of the following insecticides:

n-butyl carbitol thiocyanate (Lethane B 71)
chlorinated camphene (Toxaphene)
diethyl-*p*-nitrophenyl thionophosphate (Parathion)
1, 2, 3, 4, 5, 6-hexachloro-cyclohexane 92% gamma isomer or benzene hexachloride (Lindane or Gammexane)
1, 2, 3, 4, 10, 10-hexachloro-1 : 4, 5 : 8-diendomethano-1, 4, 4a, 5, 8, 8a-hexahydronaphthalene (Aldrin)
isobornyl thiocyanoacetate 80%, remainder related thiocyanoacetates (Thanite)
lauryl thiocyanate (Loro)
1, 2, 4, 5, 6, 7, 8, 8a-octachloro-4, 7-methano-3a, 4, 7, 7a-tetrahydroindane 60% with related dicyclopentadiene derivatives (Chlordane)

The following insect repellents were tested:

dimethyl phthalate
2-ethyl hexanediol-1, 3 (Rutgers 612)

From the meager structural evidence on insecticidal action, highly chlorinated organic molecules appear effective as nerve poisons, organic thiocyanates exert rapid depressant effects, and narcotic vapors such as carbon disulfide impart anaesthetic action. Thus tests were made on structurally related compounds. Halogenated compounds tested were:

alpha-chloronaphthalene
p-chlorophenyl isocyanate
2, 3-dichloro-1, 4-naphthoquinone
2, 4-dichlorophenol
p-fluorobenzonitrile
hexachlorobutadiene
phenacyl bromide
phenacyl chloride
sodium hypochlorite (Chlorox)
sodium pentachlorophenate
thiocyanic acid 5, 5-trichloro amyl ester
trifluoroacetic acid

Cyanates, thiocyanates and isothiocyanates examined were:

allyl isothiocyanate
ammonium thiocyanate
barium thiocyanate
phenyl isocyanate
phenyl isothiocyanate
potassium cyanate
potassium thiocyanate

The actions of other organic sulfur compounds noted were:

allyl thiourea
1, 2-ethane dithiol
ethyl mercaptan
hexamethylene dithiol
potassium ethyl xanthate
β, β'-thiodipropionitrile

The following compounds, analogous to repellents, were also tested:

dibutyl phthalate
cyclohexanol

b. *Substances irritating or toxic to mammals*

A few compounds known to be toxic to mammals and several rated as repulsive or irritating have been tested. These were:

allyl isothiocyanate
allyl mercaptan
barium thiocyanate
caffeine
carbon disulfide
copper salts (copper chloride, sulfate and acetate)
diethylamine
dimethyl sulfate
ethyl mercaptan
isovaleric acid
methyl strychnine
Parathion
phenacyl bromide
phenacyl chloride
phenyl isocyanate
phenyl isothiocyanate
phenol
piperidine
pyridine
quinine
skatole
sodium cyanide
sodium pentachlorophenate
strychnine
thallium sulfate

c. *Piscicides and fish repellents*

As mentioned previously, relatively scant attention has been given to the subject of reactions of fish to chemicals except for certain special purposes. The same or similar compounds found effective as piscicides or repellents by others were tested. These were:

acetic acid
benzoyl peroxide
cupric acetate
cupric chloride
cupric sulfate
ethyl mercaptan
hydrogen peroxide
phenol
rotenone (Fish-Tox)
sodium hypochlorite (Chlorox)
4-tertiary butyl catechol

d. *Special irritants*

The following chemicals were used to test the effects of active oxygen as a possible irritant:

sodium perborate
sodium peroxide

Reactions to reduced oxygen tension were tested by adding sodium bisulfite to the water.

Methods

Preparation of solutions

Solutions of solids were prepared by weighing, and of liquids by volumetric measurement to give the required concentration (20 p.p.m., 10 p.p.m., etc.) when diluted and mixed in the known volume of sea water in the test aquaria. Solutions were made up in sea water to a volume of approximately 100 cc. Water soluble

substances presented no difficulty, but for insoluble substances it was necessary to add about one drop of a dispersing agent before emulsifying with a hand homogenizer. In the early phase of the work Aerosol OT and Triton B 1956 were used as emulsifiers and dispersers for chemicals. Later a systematic search for a suitable general disperser for insoluble substances in sea water was made. Fifty commercial products submitted by the producing companies were tested for their ability to disperse mineral oil in sea water. Efficiency and general desirability were judged on the basis of the amount of foaming and the degree of precipitation or creaming after set intervals. As a result of these tests "Tergitol" Dispersant NPX was considered the most suitable under our conditions of testing. However, we are aware that a good disperser for mineral oil in sea water may not be the most satisfactory for other substances in the same medium. Final decision on emulsifiers and chemical dispersers will be made by testing them with specific compounds found to arouse the desired response in the test animals.

For preparation of solutions more dilute than 10.0 p.p.m. a definite volume of a more concentrated solution was taken, then an aliquot was introduced into the test aquaria. Compounds were screened initially at 20.0 p.p.m., and chemicals which were definitely effective at that concentration were tested at lower values. Although the common chemical sensory receptors of fish theoretically should be stimulated with appropriate chemicals at a maximum dilution of 0.3 to 0.4 p.p.m., the minimum dilution value of 20.0 p.p.m. was selected for initial screening to assure response by the fish if the substance had but slight stimulating value. Although such substances may exert no effect on fish in greater dilutions, final appraisal of the molecular structure for all active chemicals may disclose certain common characteristics which would lead us to more effective irritants and repellents, and ultimately aid in framing a structural basis of activity.

The minimum time of testing was two minutes, and observations were generally concluded at the end of that time unless the fish responded to the chemical. Although this brief interval may seem inadequate for results of such tests, it is in line with the objectives of the problem—to find a rapidly acting substance capable of dispersing schooling fish.

Apparatus

The initial screening of chemical substances was conducted in aquaria of 50 liters capacity containing a school of four or five small fish, *Kuhlia sandvicensis,* each ranging from 30 to 60 millimeters in total length. The outer surface of the rear glass side of each aquarium was painted black to effect greater contrast with the silvery fish on movie film. An electrically driven stirrer was mounted in the corner of each tank to hasten the dispersal of chemical substances throughout the tank. For recording the speed of action an electric clock with an enlarged second hand was mounted above each tank. Overhead a battery of three Photoflood lamps in aluminum reflectors illuminated the contents of each aquarium.

The introduction of the chemical substance and the subsequent response on the part of the fish were permanently recorded on 16 mm. black-and-white movie film. Visual observations on behavior were also recorded. The movie record was made to detect subtle differences in reaction difficult to describe verbally or to remember for subsequent comparison.

Results

Inactive chemicals

The following list of chemicals elicited no observable response at a concentration of 20.0 p.p.m.:

acetonitrile
allyl thiourea
ammonium thiocyanate
barium thiocyanate
benzoid acid-*o*-(alpha mercaptoacetamido)
benzoyl peroxide
catechol
chlorinated camphene (Toxaphene)
alpha-chloronaphthalene
p-chlorophenyl isocyanate
cupric chloride
cupric sulfate
cyclohexanol
cyclohexyl-4-amino phenol HCl
gamma-chlorobutyronitrile
2-3-dichloro-1, 4-naphthoquinone
diethylamine
dimethyl phthalate
dioxane
ethylene oxide
ethylene oxide polymer
p-fluorobenzonitrile
hexamethylene dithiol
hydrogen peroxide
hydroquinone
β-β'-iminodipropionitrile
indole-3-acetic acid
methyl acrylate
methyl strychnine
mica (500 mesh)
morpholine
1, 2, 4, 5, 6, 7, 8, 8a-octachloro-4-7-methano-3a, 4, 7, 7a-tetra hydroindane (Chlordane)
piperidine
potassium cyanate
potassium ethyl xanthate
potassium thiocyanate
pyridine
Rutgers 612
skatole
sodium perborate
sodium peroxide
thallium sulfate
β, β'-thiodipropionitrile
triethanol amine
urea

Active chemicals

Active chemicals and the degree of response by the test animals at various dilutions are summarized in Table I.

Effects of particle size and true solution

Since many of the effective chemical compounds were insoluble in sea water and had to be dispersed therein in the form of an emulsion, it was necessary to know something about the relation of droplet size to the magnitude of the response evoked in the fish. Although no data are available relating the intensity of response by fish to the comparative number of sensory receptors stimulated by substances dissolved or suspended in water, we are probably permitted to assume *a priori* that the magnitude of response varies directly with the number of receptors stimulated. Thus, when fish are exposed to dilutions of relatively insoluble substances in the neighborhood of 0.1 p.p.m. it is quite possible that the number of molecules in solution or the number of particles in suspension bear significantly on the intensity of the response.

To illuminate this question for purposes of preliminary evaluation and judgment of the most potent compounds tested, the effect of particle size and true solution on the response to one of the most irritating chemicals discovered (allyl isothiocyanate) was measured. Three preparations were made. First, the dispersing agent, "Tergitol" Dispersant NPX, was added to the chemical as was sea water, followed by simple emulsification in a hand-operated homogenizer. Second, the chemical was placed in isopropyl alcohol to form a true solution to which "Tergitol" Dispersant NPX and sea water were added before emulsifying in a homogenizer. Third, a true solution of the chemical was formed by adding sea water and warming until the insoluble phase had disappeared. Droplet size of the first two emulsions was measured microscopically using an ocular micrometer. In the first suspension the droplets were quite variable in diameter, averaging 4.5 μ, whereas in the second suspension they were of quite uniform size, averaging 3.0 μ.

Tests were conducted as described previously using dilutions of 0.1 p.p.m. for each of the three preparations. No detectable difference in response was discerni-

TABLE I

*Summary of the responses of Kuhlia sandvicensis to various dilutions of chemical irritants. The symbol *** denotes a violent reaction, ** a medium reaction, * a slight reaction, and — no discernible reaction. See text for details.*

Chemical	Emulsifier	Dilution in p.p.m.						
		20.0	10.0	2.0	1.0	0.2	0.1	0.05
Acetic acid	None	*						
Acrylonitrile	None	*						
Allyl isothiocyanate	None	***			***	***	**	—
Allyl mercaptan	Tergitol NPX	*						
Benzene	Triton B 1956	*						
n-Butyl carbitol thiocyanate	None	***			—			
Caffeine	Aerosol OT	*						
Carbon disulfide	None	*						
Catechol (derivative)	Aerosol OT	*						
alpha-Chloronaphthalene	Aerosol OT	*						
Cupric acetate	None	*						
Dibutyl phthalate	Aerosol OT	*						
2,4-Dichlorophenol	Dioxane and Triton B 1956	**						
Diethyl-*p*-nitrophenyl thionophosphate (Parathion)	Aerosol OT	**						
Dimethyl sulfate	None	*						
2, 4-Dinitronaphthol-ammonium salt	None	*						
Du Pont PB-70	Dioxane and Triton B 1956	*						
1, 2-Ethane-dithiol	Triton B 1956	**						
Ethyl mercaptan	None	***		—	—			
Hexachlorobutadiene	Aerosol OT and Triton B 1956	*						
1, 2, 3, 4, 5, 6-Hexachlorocyclohexane, 92% gamma isomer (Lindane)	None	***			*			

TABLE I—*Continued*

Chemical	Emulsifier	Dilution in p.p.m.						
		20.0	10.0	2.0	1.0	0.2	0.1	0.05
1, 2, 3, 4, 10, 10-Hexachloro-1:4, 5:8-diendomethano-1, 4, 4a, 5, 8, 8a-hexahydronaphthalene (Aldrin)	None	***		—	—			
Hydroquinone	None	*						
2-Hydroxy-3-cyclo-hexyl-1, 4-naphthoquinone	Benzene and Aerosol OT	**		*				
Isobornyl thiocyanoacetate (Thanite)	None	***	***	***	***	*		
Isovaleric acid	None	*						
Lauryl thiocyanate (Loro)	None	***						
Methyl methacrylate	None	**						
Phenol	None	***		**				
Phenacyl bromide	Ethyl alcohol and Tergitol				***		**	—
Phenacyl chloride	Ethyl alcohol and Tergitol				***		**	—
Phenyl isocyanate	Aerosol OT	**						
Phenyl isothiocyanate	None	***	***		*			
Potassium thiocyanate	None	*						
Quinine	Ethyl alcohol and Tergitol		*					
Rotenone (Fish-Tox)	None	**			—			
Sodium bisulfite	None	**	—					
Sodium cyanide	None	***		***	*			
Sodium hypochlorite (Chlorox)	None		***	***		—		
Sodium pentachlorophenate	None	**						
Strychnine	Ethyl alcohol and Tergitol		*					
4-Tertiary butyl catechol	Aerosol OT	**	—					
Thiocyanic acid 5, 5, 5-trichloro amyl ester	Tergitol		***	**				
Trifluoro acetic acid	None	*						

ble with the two suspensions, indicating, possibly, that differences in particle size of the magnitude mentioned do not make any appreciable difference in the reception and response to stimuli. However, the true solution did evoke a discernibly greater activity, thus possibly implying that in such dilutions molecular or near-molecular size of the irritant contributes to more widespread stimulation of available receptors.

DISCUSSION

Intensity, nature and rapidity of responses

Behavior patterns of *Kuhlia sandvicensis* in response to chemical stimulation, mediated primarily through the common chemical sensory receptors, permitted analysis by three separate criteria: (1) the intensity of response (slight, medium or violent, as indicated by the asterisks in Table I), (2) the nature of the response (the particular nervous receptors stimulated and the sensations aroused), and (3)

the intervals between bodily contact with the chemical substance and the first discernible reaction. Precise quantitative analyses of the intensity of responses are impossible to achieve, but satisfactory estimates were made on a background of many tests evoking a wide variety of behaviorisms. Slight responses elicited by mild irritants were manifested by rapid mouth movements, efforts to avoid the substance, and vertical swimming, first to the surface, then down, repeated rapidly. These reactions to slight irritants occurred invariably, together with other activity, in responses rated as medium and violent, and were usually the prelude to these intensified reactions. A medium response was denoted by various patterns of behavior all more intense than that indicated for slight response, but in no case did the substances arouse violent reaction. Rapid swimming vertically and about the aquarium, gulping at the surface, jerky motion, etc., were types of responses that rated this designation. Extreme behavior of several characteristic types was designated as violent response. Very erratic and rapid swimming, often leaping out of the aquarium, paralysis, head shaking, blindness and death within two or three minutes were classified in this category.

The mode and place of action of chemicals on fish are not simple to define. The widespread distribution of common chemical sensory receptors on the general body surface, eyes, nasal capsules and the mouth, coupled with the fact that the complexes of chemical senses are not well differentiated for fish, behavioristically speaking, as they are for mammals, precludes the expectation of precise patterns of response to particular stimuli. Excluding the chemical senses of taste and smell, we would still expect different modalities of the common chemical sense in fish just as are found in humans, where different sensations are experienced from substances attacking the eyes, the nose or the throat. An analysis of the responses by fish to chemicals known to arouse particular sensations in other animals bears out this supposition. For example, well known general skin irritants for man such as phenol and allyl isothiocyanate apparently stimulate all free nerve endings on the body making it impossible to pinpoint a site of irritation because of the violent reaction evoked. Also, the very potent lachrymators, phenacyl bromide and phenacyl chloride, arouse intense sensations resulting in violent head shaking and early indications of blindness, precisely the mode of response expected for substances irritating primarily to the eyes. Sodium cyanide and thiocyanic acid 5, 5, 5-trichloro amyl ester, substances related to respiratory poisons for insects (Brown, 1951), and the reducing agent, sodium bisulfite, caused fish to stay at the surface where they gulped air and showed other typical signs of suffocation. Both tetanic and flaccid paralytic effects were noted for certain compounds tested. A clear example of tetanic paralysis wherein the operculum was fixed rigidly at an acute angle on the head, the fins held stiffly outward, and the body bent in a convulsive manner resulted from the introduction of isobornyl thiocyanoacetate. This substance is known to act as a narcotic for insects but induces paralysis in mammals. Ethyl mercaptan, of which little is known neurophysiologically except for its effect on the olfactory receptors, also induced a severe tetanic paralysis in fish. Notable among those substances inducing flaccid paralysis was thiocyanic acid 5, 5, 5-trichloro amyl ester, which, in addition, destroys the equilibrium. The slowly moving fish gradually leave the surface, where they also exhibit symptoms of suffocation, and swim upside down before they eventually become moribund on the bottom of the tank.

Characteristic responses which appeared to differentiate between stimulation of taste receptors and of the common chemical sense were aroused by testing quinine and strychnine. These substances, according to Scholl and Munch (1937), are two of the most bitter substances stimulating the human taste, strychnine being about three times as bitter as quinine. Brucine, about three to four times as bitter as strychnine, was not available for testing. Test fish shook their heads violently and made "spitting" motions for a few seconds after the initial contact with the alkaloids, then, exhibiting no irritation, settled down to normal schooling behavior. Quinine actually evoked a more intense and more lengthy response than strychnine.

Comparative responses of fish and other animals to chemical irritants

Good examples of the highly selective nature of the sensations aroused by chemicals on different organisms are clearly shown in the list of inactive chemicals and in the table of active compounds. Although there was some relationship between chemicals irritating to fish and those having a similar effect on other animals, there were many and significant exceptions.

Exceptions in the ranking position of the chemicals tested thus far were the high molecular weight thiocyanates which had little or no effect on the olfactory and common chemical sensory receptors of mammals, and may even have a relatively low order of toxicity, but are classed here as some of the most effective irritants to fish. A possible explanation for this disparity in reactivity may be associated with the medium in which the animals exist, air in the case of mammals and water in the case of fish. In the former, where air transport is necessary, the relatively low vapor pressure would seem to be a plausible explanation of the lack of response. In the latter, greater reactivity for fish probably results from relatively greater solubility, an assumption which awaits confirmation. Substances of this character emphasize the necessity of an empirical search through a wide variety of chemical types for compounds which, although not irritating to other animals, may arouse intense sensations in fish.

An appreciable number of the chemical groups tested were selected because they elicit a profound and often violent response in man, but we have found them rather ineffective as fish irritants. Such chemicals as most mercaptans, certain amines, some nitriles and skatole fell into this category. For an explanation of much of this disparity in reaction we concur with Moncrieff (1944) that in man conditioned reflexes are important, resulting from associating many of these odors with unpleasant experiences or with substances which we learn to recognize as toxic. Significant also, we suspect, is the fact that most of these substances have a low solubility in sea water, thus having a sub-minimal concentration for reaction, whereas nearly all have an appreciable vapor pressure.

Substances which were found to have about equal effect in irritating fish and mammals fall generally into the groups of intense skin irritants and lachrymators, examples of which are allyl isothiocyanate, phenacyl chloride and phenacyl bromide. Noteworthy among these generally effective compounds is the prevalence of sulfur, which lends support to the point stressed by Moncrieff (1944) that this element imparts skin-penetrative power, thus increasing the vulnerability of the free nerve endings. Our results also emphasize the importance of the bond type, whether it be ionic or covalent, in governing the effect of irritants. For example, all thio-

cyanates tested were effective irritants in the organic covalent form, but the ionic salts of ammonium, barium and potassium thiocyanate were ineffective. Conversely, covalent cyanides or nitriles proved ineffective, but the cyanide ion belonged with the group of irritants.

Substances found most effective in repelling sharks and certain bony fishes (Burden, 1945; Whitley and Payne, 1947), cupric acetate and acetic acid, were only mildly irritating to our test species and show little promise of satisfying the objectives of this study.

It was pointed out previously that too few data were available in the literature to formulate conclusions on comparative stimulus-response activity in the chemical senses between humans and fish, but that the ratio of response to strength of stimulus for the complex of chemical senses in humans was rather well known, especially since the researches of Katz and Talbert (1930). A hypothetical working parallelism for the chemical senses in fish was established on the basis of the maximum detectable dilution of a chemical substance in fish mediated through the olfactory receptors, based on the results of Hasler and Wisby (1949). We calculated, in accord with the proportional stimulus-response data for the chemical senses in man, that the eyes of fish might possibly be irritated with suitable chemicals at a dilution of about 0.08 p.p.m., and that the common chemical sensory receptors over the remainder of the body might be stimulated at a maximum dilution of about 0.3 or 0.4 p.p.m.

Our data indicate that both phenacyl bromide and phenacyl chloride, known to be powerful lachrymators and to induce behavior in our experimental animals suggestive of intense irritation of the eyes, were quite effective in a dilution of 0.1 p.p.m. and ceased to be discernibly effective at 0.05 p.p.m., precisely fitting our hypothesis. The most effective general irritant, allyl isothiocyanate, evoked observable non-localized responses at the level indicated for the two lachrymators just discussed. The next most effective general skin irritant, isobornyl thiocyanoacetate, failed to arouse discernible sensations at dilutions below 0.2 p.p.m.

Solubility of organic compounds in sea water

Our results have pointed to the fact that the distribution of the reacting substances in sea water, either in the form of a true solution or a colloidal sol, is basic to stimulus-response investigations of this type. These data, although only suggestive in nature, seemed to indicate that finely dispersed particles, particularly in the less concentrated solutions, were not as effective for the stimulation of the free nerve endings over the general body surface as molecules in a true solution. Clouding this aspect of the work is the near absence of solubility data for organic compounds in sea water. However, we can extend what is known about the solubility of these compounds in pure water in an effort to predict their behavior in sea water. It is to be expected that solubility will be generally less in sea water because of the "salting out" effect. Moreover, we would expect in any group of organic compounds having closely related structural composition that the solubility would be greater for those of lower molecular weight and of greater polarity. Considerations such as these guided our selection of compounds.

Observations on the use of chemical dispersing agents with rather insoluble compounds indicated, because of the persistence of a separate phase of emulsion

droplets in very dilute solutions where the concentration was well below the amount capable of existing in true solution, that the film of emulsifying agent surrounding each droplet may inhibit the attainment of equilibrium between the two phases of solvent and solute. Because of this, caution must be used in selecting and using chemical dispersing agents when it is desired to achieve maximum molecular concentration of the solute.

General conclusions of a preliminary nature

Although generalizations at this stage of development in this project may be somewhat premature, certain aspects appear reasonably clear. An evaluation of the behavior of the test schools in response to the active chemicals definitely indicated that substances perceived only through the human chemical receptors as odors or tastes did not arouse sensations capable of dispersing schooling fish, but that substances which stimulate human receptors for the common chemical sense, such as general skin irritants, lachrymators and nerve poisons, were effective. Thus future work will be directed toward securing more potent irritants and toward developing synergistic reactions with those already proven effective. A listing of the types of compounds expected to be most effective, based on our analysis of eighty-seven chemicals tested thus far, and placed in order of probable importance, includes multi-halogenated organic compounds, organic thiocyanates, organic isothiocyanates and halogenated ketones. Revisions of this list may be expected as further tests are made within each group, as our list of groups tested is extended, and as other species of schooling fish are used as test animals. We wish to point out again that, although the results of this investigation have been secured by testing and analyzing the reaction of fish to 87 judiciously selected compounds, these appraisals are considered preliminary because of the vast amount of work yet to be done along the lines suggested by this phase of the investigation.

The authors are indebted to many chemical companies which have offered chemical dispersing agents and chemical samples for testing and greatly appreciated advice.

Summary

1. The objective of this study was to find, by judicious selection and testing, whether or not certain chemical compounds in exceedingly dilute concentrations would arouse sensations in fish which would alter the sensory bonds between them, thus effecting dispersal of schooling fish.

2. The basic considerations in selecting chemicals capable of dispersing schools of fish were (1) that they be grossly irritating to fish rather than merely toxic or narcotic, and (2) that they be rapid in action, reaching, in our experiments, a maximum effectiveness within a period of two or three minutes.

3. A preliminary classification of the 87 compounds tested included insecticides and insect repellents, substances irritating or toxic to mammals, piscicides and fish repellents, and special irritants. Suitable chemical dispersers were added to the relatively insoluble compounds. Tests were made at dilutions of 20.0, 10.0, 2.0, 1.0, 0.2, 0.1, 0.05 p.p.m.

4. The intensity, nature and rapidity of response to introduced chemicals were recorded by observation and by motion pictures. Although a clear perceptual pattern of the mode and site of action of certain chemicals known to arouse localized sensations in terrestrial animals was not definitely discernible, certain reactions suggested that special areas were intensely stimulated. General skin irritants (phenol, allyl isothiocyanate) strongly stimulated the entire body; lachrymators (phenacyl chloride, phenacyl bromide) seriously irritated the eyes and impaired vision; some general nerve poisons (isobornyl thiocyanoacetate, ethyl mercaptan) induced tetanic paralysis; whereas another (thiocyanic acid 5, 5, 5-trichloro amyl ester) resulted in flaccid paralysis; and respiratory impairment was brought about by a reducing agent (sodium bisulfite) and by probable respiratory poisons (sodium cyanide, thiocyanic acid 5, 5, 5-trichloro amyl ester).

5. Examples of the highly selective nature of the sensations aroused by chemicals on different organisms were clearly demonstrated. High molecular weight thiocyanates which have little or no effect on the common chemical sense of mammals are highly effective irritants for fish. Others (mercaptans, certain amines, some nitriles, skatole), exceedingly irritating to man, appear to have little or no effect on fish. Substances classed as intense skin irritants or as lachrymators (allyl isothiocyanate, phenacyl chloride, phenacyl bromide) for man appear to be equally effective for fish. Underlying reasons for such disparities and similarities in reaction were suggested.

6. Certain characteristics of molecules appear to be significant in predicting success for irritants, and thus contribute toward a framework for a theory establishing relations between chemical constitution and physical properties on the one hand and aggressive action on the other. Increased aggressiveness with sulfur in the molecule was noted, and the bond type, ionic or covalent, apparently exerts a governing effect with irritants as shown by contrast between the very effective covalent thiocyanates and the ineffective ionic thiocyanate salts.

7. A striking proportionate parallelism seems to exist for stimulus-response reactions of all the chemical senses between humans and the fish used for these tests.

8. Results thus far indicate that substances perceived as odors and tastes for humans do not arouse sensations capable of dispersing fish in schools, but that general skin irritants, lachrymators and nerve poisons may be capable of doing so. Types of compounds which now seem most effective for dispersing fish in schools are multi-halogenated organic compounds, organic thiocyanates, organic isothiocyanates and halogenated ketones.

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STUDIES ON THE ELASMOBRANCH KIDNEY. II. REABSORPTION OF UREA BY THE SMOOTH DOGFISH, MUSTELUS CANIS

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There has been evidence of reabsorption of urea by the elasmobranch tubules for over forty years. The fact that the blood and tissues contain an unusually high concentration of urea has been known since 1859, and the discovery that the urine contains a much lower level of this material was made as early as 1906. There is, therefore, a wide variation in the way in which the various kidneys treat urea: in the mammal, a back diffusion, perhaps with other factors involved (Shannon, 1936); in the aglomerular fish, probably an inward diffusion (Marshall, 1930); in the frog, secretion (Marshall, 1932; Walker and Hudson, 1937); and in the elasmobranchs, active reabsorption.

There appears to have been no adequate inquiry concerning the detailed behavior of the elasmobranch kidney in relation to urea, despite the long existence of evidence of its reabsorption and its quantitative demonstration by Clark and Smith (1932).

There has been no demonstration of the site of urea reabsorption. It was suggested by Marshall (1930) and by Smith (1931) that the "special segment" of the elasmobranch tubule is responsible for urea reabsorption. This has been accepted by Baldwin (1937) and other writers. However it has been shown by direct puncture methods that this special segment does not exist in the tubule of the spiny dogfish, *Squalus acanthias* (Kempton, 1943). Moreover there is reason to believe that the concept of a special segment is a mistake and that the segment does not exist in any elasmobranch tubule.

Attention now has been turned to a study of urea reabsorption by the entire kidney with the hope that such an investigation might give some clue concerning the reabsorptive mechanism. This primary objective was successful to the extent that some of the factors controlling the rate of urea reabsorption have been revealed. Conclusive evidence as to the site of reabsorption in relation to other tubule functions has not been found.

Materials and Methods

The simultaneous reabsorption of water, urea and glucose has been studied, using inulin as a measure of glomerular filtration. Clearances were calculated by use of the well-known method of Smith (1939): UV/P, in which U is the mgm.% of the material in the urine, V is the volume of urine in cc./hr./k., and P is the mgm.% present in the plasma. Contrary to the usual practice in urea studies, plasma concentrations instead of whole blood were used because of the finding by Dr. Arthur K. Parpart (personal communication) that the erythrocytes of this species are only slowly permeable to urea.

For inulin determinations the resorcinol method of Hubbard and Loomis (1942) was employed, with two modifications. Since it was more convenient to use larger cuvettes in the spectrophotometer, 2.0 cc. samples of the plasma or urine filtrate diluted to the range of the method were used, with the final dilution in sugar tubes to the 12.5 cc. level. Heating was extended from 8 to 16 minutes, a change which produced somewhat more constant results. The tubes were read against a blank of the reagents in distilled water. Use of other blanks recommended by Hubbard and Loomis was tried repeatedly, but since no effect was ever found their use was dropped in the routine analyses. The method as modified was found to be very satisfactory, and it was not affected even by elevated glucose levels. Special tests indicated that interference from this source did not appear until the glucose level in the diluted plasma or urine filtrate was raised to about ten times the concentration of inulin.

The urea method was a slight modification of the urease and direct Nesslerization method of Koch and Hanke (1948). Because this method was developed for the relatively low plasma level and the high urine level of mammals, several changes had to be introduced to care for the reversed situation found in the elasmobranchs. Plasma was first diluted to range (usually a 20 × dilution) and then digested with Koch's glycerol urease extract at 50° C. for 60 minutes. In preparing the filtrate, the sodium tungstate and sulfuric acid were reduced in amount to 0.5 cc. each, and the normality of the acid was raised to 1.0. After filtration and Nesslerization, the readings were made against ammonium sulfate standards. Urine was treated by the same method, except that the initial dilution was usually 6 ×. The method as used includes any ammonia nitrogen which might be present. It was deemed inadvisable to attempt to separate the two sources of nitrogen because there might be some urea decomposition during the prolonged collection periods, and in addition Denis (1922) found very little ammonia nitrogen in elasmobranchs.

The method as modified was satisfactory for the purpose, but it had two objectionable features: the high dilution magnified very greatly any differences in the spectrophotometric readings; for some reason a blank containing urease gave an undue amount of light scatter. In a few cases the urine samples also gave excess scatter and had to be discarded. The source of this effect was not determined.

For glucose analyses the arsenomolybdate method of Nelson (1944) was selected. Undiluted plasma filtrates and diluted (4 ×) unfiltered urine were used. The blank consisted of the normal reagents in distilled water.

All readings were made with a Coleman Junior Spectrophotometer with cuvettes 19 mm. in diameter. Wave-lengths used were as follows: inulin method, 495 millimicra; urea, 560; glucose, 500.

Only female dogfish were used. This was due partly to the small number of males actually caught, and partly to possible complications resulting from the use of the same ducts for seminal fluid and urine. The weight of the experimental fish ranged from 5.3 to 10.3 kilograms. Below this range the urinary papilla was so small that urine collections were impracticable; at the top weight the animals were so strong that it was barely possible to handle them without damage to the balloon which collected the urine. During May and June animals of the necessary size were obtained from the traps of commercial fishermen, but after about the first of July it was necessary to catch them by hook after sunset either in deep water by the shore or from a boat in the deeper water of the harbor. The fish were always

transferred immediately to a tank of fresh sea water and rushed to a live car in a float, by which tidal currents swept at a rate sufficient to ensure rapid and complete change of water (or, in 1950, to a large concrete tank with well aerated running water). The animals refused even living food in captivity; in fact they were very likely to regurgitate food which had been taken before capture. In a few cases the hook wound continued to bleed, especially when the animals were handled for collecting blood and urine. (These animals were discarded.) At best the life in captivity was limited to a few days, and there was some evidence that this was in part a temperature effect. As the water temperature became higher during the summer their life expectancy became reduced. The warmer waters of the Eel Pond in mid-summer killed dogfish in a few hours. A period of exceptionally hot weather had a very deleterious effect on the animals in use at the time. The more constant temperature in the concrete tank allowed the animals to remain in good condition for several days longer than when the live car was used. Usually the experiment was ended when the urinary papilla became so damaged that it would no longer support a catheter. The animals usually lived several days after the enforced termination of the collections. It was only in the most prolonged experiments that there was any appreciable reduction in the blood sugar level.

The animals were prepared for the collection of urine by the removal of the tip of the urinary papilla, and the insertion into the papilla of an in-lying catheter of glass. A rubber balloon, tied securely to glass tubing, was anchored by ligature to the ventral surface of the tail. The catheter was connected with the glass tubing by a piece of flexible rubber tubing, with sufficient slack to permit swimming and movements of the cloacal region without placing a strain on the catheter and the papilla. At the end of each collection period, the ligature holding the balloon was cut, the tubing disconnected from the catheter, and the urine poured into a collecting vessel. The abdomen was massaged carefully to force from the kidney ducts any fluid they might contain, this urine being removed from the catheter by syringe. The amount obtained by massage usually varied from zero to five cubic centimeters, although in one very exceptional case 40 cc. was collected. The balloon was then replaced by a fresh one. These balloons were of sufficient size to ensure that no back-pressure developed. These and other manipulations were performed on the float containing the live car, or beside the concrete tank. This made it possible to transfer the animal quickly from the circulating water to a trough, where it was tied on its back with its head immersed in fresh sea water. No anesthetic was ever used.

Administration of various materials was by diverse routes. Inulin, prepared as a 20% solution in warm distilled water, was injected intravenously through the caudal blood vessel. In some experiments other materials were also injected in the same way. Water was sometimes given by stomach tube, but this was not the standard procedure. Urea was administered in three different ways. Intravenous injections were fatal quickly. In a number of cases intraperitoneal injections were also fatal, but more slowly, with the liver exhibiting severe lesions. Intramuscular injections were partially successful, since an increase of plasma urea levels ranging up to 50% was obtained in about half of the injected animals. The firm unyielding nature of the flesh made difficult the injection of more than a few cubic centimeters at any one point. It was necessary therefore to make a series of small injections along the epaxial muscles of both sides. The dosage was 10 cc. per kilogram of a

50% solution in distilled water, freshly prepared at room temperature. In some cases this dosage was repeated twice, giving a total urea administration of 15 grams of urea per kilogram of body weight.

Blood was collected by syringe in amounts of 10 cc., and was transferred at once to test tubes containing 0.1 mgm. potassium oxalate, precipitated by drying from a

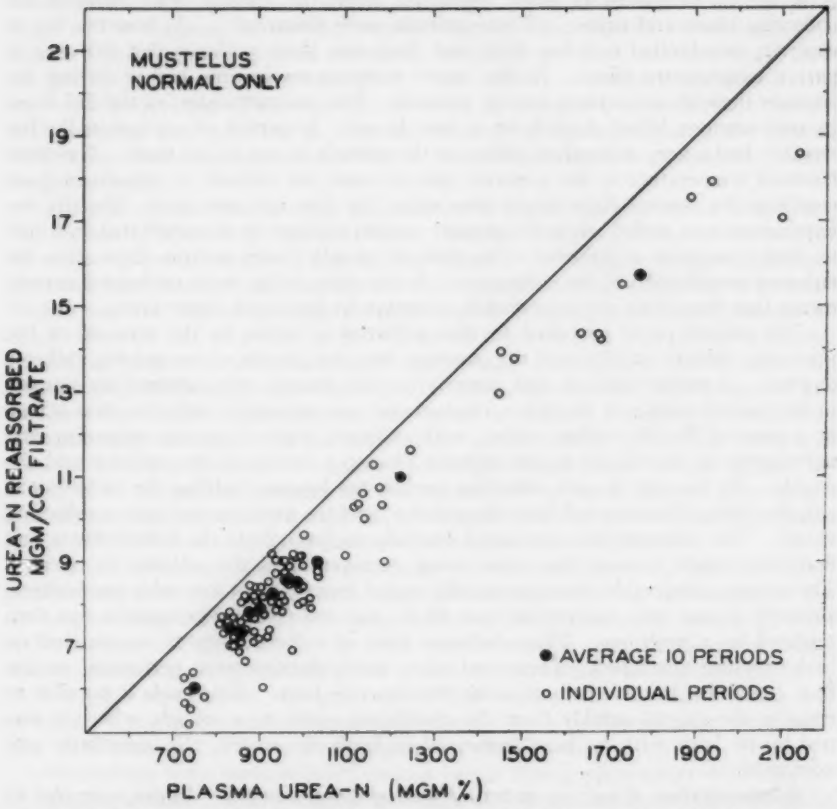

FIGURE 1. Relationship between the reabsorption of urea from each cubic centimeter of filtrate and the plasma urea level; 110 collection periods from smooth dogfish in good condition. The abscissa could be labelled equally well "Filtrate urea-N (mgm.%)" instead of referring to plasma level. For each point, the vertical distance to the diagonal line represents the unreabsorbed moiety; the vertical distance extrapolated to a zero baseline represents the reabsorbed urea.

20% solution. Upon return to the laboratory from the float or tank this oxalated blood was centrifuged immediately, the plasma either being used at once or being stored over night in a very cold refrigerator. When haematocrit readings were made, capillary tubing of uniform internal diameter of 0.6 mm. was used, the centrifugation being for one-half hour in an International clinical centrifuge using

direct current. Top speed of 3000 r.p.m. gave a centrifugal force of 1600 × gravity. This force, for this period of time, gives as complete packing as an air turbine developing 20,000 × gravity (according to turbine determinations by Dr. James W. Green).

The routine plan of the experiments, from which there was considerable deviation, called for the capture of the fish one evening, its weighing the next morning, the injection of inulin early that afternoon, and the start of the first collecting period the next morning. Collections of blood were made at the beginning and end of each period. Urine was collected at the end of the period. Usually collections were made morning, early afternoon and evening, giving periods of approximately 12, 6, and 6 hours. In many cases a midnight collection was made also, thus dividing the day approximately into four six-hour periods. The delay between the injection of the inulin and the start of the first collection period placed all periods on a fairly flat portion of the inulin tolerance curve.

Results

There is a linear relationship between the reabsorption of urea from each cubic centimeter of filtrate and the plasma urea concentration (or the filtrate concentration). In general, regardless of the concentration of urea in the plasma and filtrate, all of the urea is reabsorbed except a constant residuum which stays in the tubule. This relationship is true on the average (Fig. 1), the minor variations in the unabsorbed urea being related to other factors as indicated below. This general relationship holds true over a range of normally occurring plasma levels in which the highest is approximately triple the lowest. Of all the data obtained, this average level of the unabsorbed urea in the tubules is the only constant factor. Raising the urea level, in an attempt to determine whether there is a definite tubular maximum at which the relationship would no longer hold, met with failure. Fortunately the range of normal variation of plasma level was sufficiently great to permit the linear relation to appear.

From *a priori* considerations there are a number of other correlations which might be expected. Surprisingly these do not appear and some of these negative results seem to have interpretative significance. Factors which show no correlation include the following combinations:

(1) Plasma urea level (mgm.%) and urea reabsorption (mgm./hr./k.)
(2) Plasma urea level (mgm.%) and urea filtered (mgm./hr./k.)
(3) Filtration rate (cc./hr./k.) and urea reabsorption (mgm./hr./k.)
(4) Filtration rate (cc./hr./k.) and urea reabsorption (mgm./cc. filtrate)
(5) Urea reabsorption (mgm./hr./k.) and glucose reabsorption (mgm./hr./k.)
(6) Inulin U/P ratio and urea U/P ratio
(7) Urea filtered (mgm./hr./k.) and urea reabsorbed (mgm./hr./k.)
(8) Urea filtered (mgm./hr./k.) and urea reabsorbed (mgm./cc. filtrate)
(9) Plasma urea level (mgm.%) and % urea reabsorbed
(10) Glucose reabsorbed (mgm./hr./k.) and urea reabsorbed (mgm./cc. filtrate)
(11) Urine urea level (mgm.%) and urea reabsorbed (mgm./cc. filtrate)

There is no reason to believe that the lack of correlation is due to incomplete emptying of the kidney ducts, although this is theoretically possible in some cases.

In the first place, every effort was made to massage all the urine from the ducts. Secondly, when the data are in terms of a unit volume of filtrate, as in 4, 8, 10 and 11 above, uncollected urine could have only a very slight effect on the calculations.

The scatter in the data is not merely fortuitous. While the main amount (average 87%) of urea reabsorption from each cubic centimeter of filtrate is correlated with the plasma (and filtrate) level, the variations in unabsorbed urea are correlated with water reabsorption (Fig. 2), urine flow (Fig. 3), and urine urea concentration (Fig. 4). In other words, the variations of unabsorbed urea are correlated with the behavior of the tubules toward water, at least in part, with more

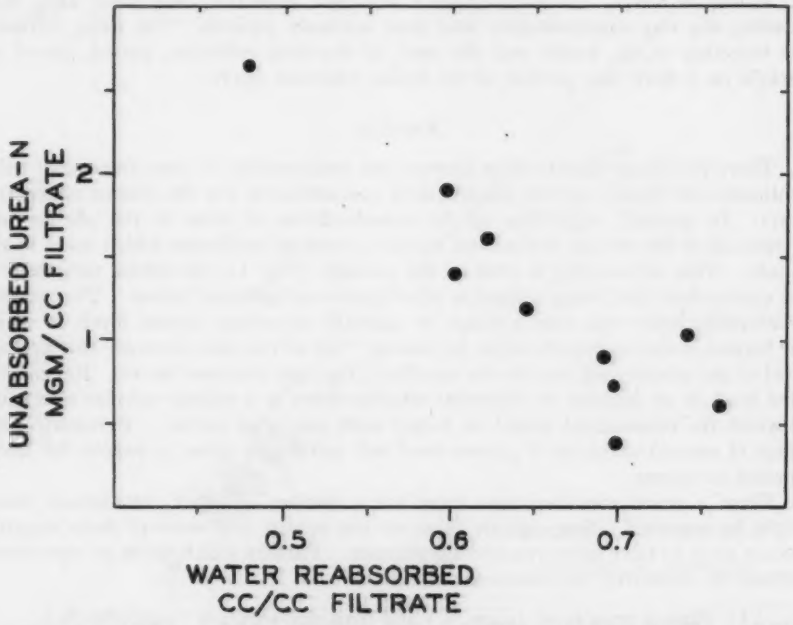

FIGURE 2. Variation in the unabsorbed urea in relation to the amount of water reabsorbed, both in terms of each cubic centimeter of filtrate. Each point represents the average of 10 collection periods.

urea remaining in the tubules unabsorbed when there is less water reabsorption, a higher urine rate, and concomitantly a higher concentration of urea in the urine. It should be pointed out that these variations in unabsorbed urea are definitely not correlated with differences in the rate of filtration, of glucose reabsorption, or the absolute rate of water or urea reabsorption. This will be referred to in the discussion.

As mentioned previously, an attempt was made to raise the urea level of the blood above the normal range. This proved to be difficult. One great obstacle was the fact that following urea injections the animals tended to continue to bleed

at every point of urea injection, and even to start bleeding at the points from which blood had been withdrawn previously. Even in the most successful experiments, the haematocrit reading fell drastically, and the blood became extremely fluid. How much of this was due to continuous seepage of blood outward, and how much, if any, was due to an inward diffusion of water through the gills, remains obscure.

The greatest increase in plasma urea-N level obtained by urea injections was from 870 mgm.% before the injection to 1230 mgm.% afterward. The highest level obtained was 1550 mgm.%, a rise from a pre-injection level of 1240 mgm.%. These new levels were well below the maximum found occurring naturally. In

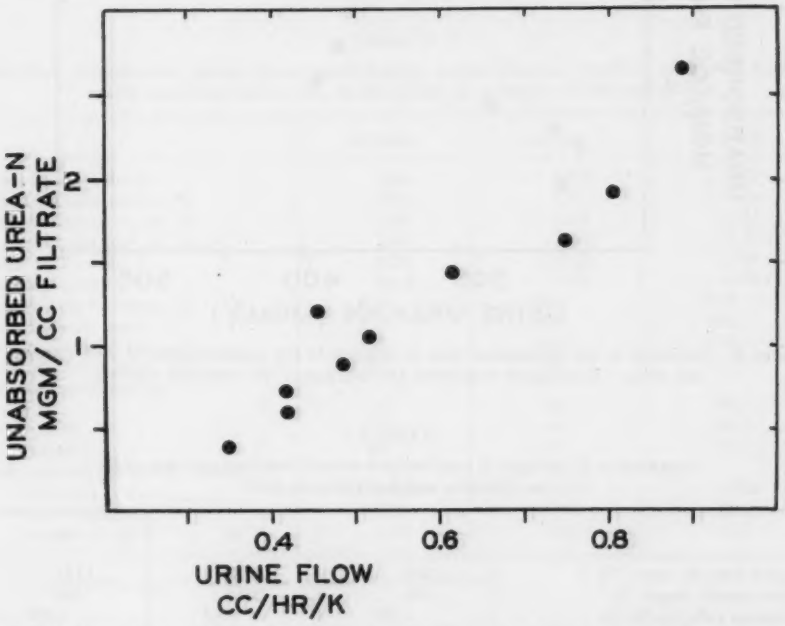

FIGURE 3. Variation in the unabsorbed urea in relation to the rate of urine flow. Each point represents the average of 10 collection periods.

these urea-injected animals there was no demonstration of a definite tubular maximum, but instead the reabsorption of urea from each cubic centimeter of filtrate was markedly depressed as compared with the uninjected animals. In preliminary results reported earlier (Kempton, 1948) the rate of reabsorption after injection was of the order of magnitude to be expected at the urea level prevailing before the injection. However, more data indicate that this was fortuitous, the rate of urea reabsorption not being "set" in relation to the pre-injection urea level.

This depression of urea reabsorption is probably without significance in relation to the reabsorptive process, because glucose and water reabsorption were similarly depressed. A comparison is given in Table I, in which the averages of the urea

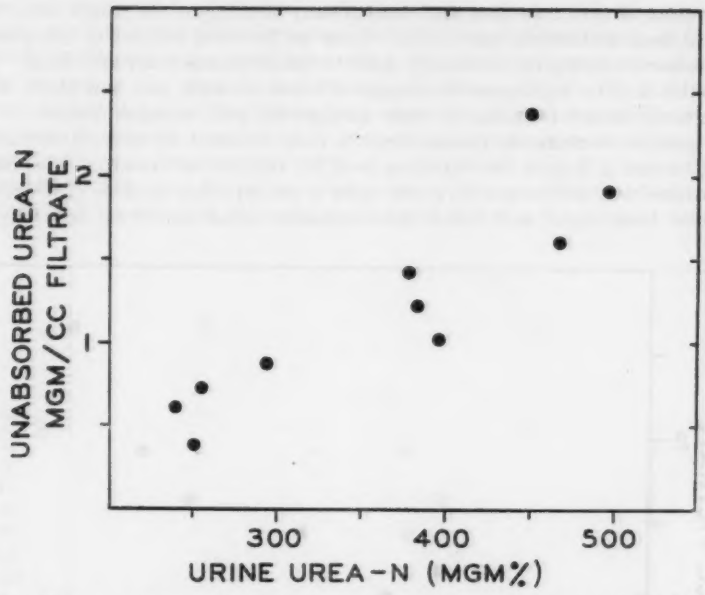

FIGURE 4. Variation in the unabsorbed urea in relation to the concentration of urea nitrogen in the urine. Each point represents the average of 10 collection periods.

TABLE I

Comparison of averages of urea-injected animals with normal ones at the pre-injection and post-injection levels

	I	II	III
Plasma urea-N, mgm. %	898	1196	1122
Urine urea-N, mgm. %	396	1083	306
Filtration rate, cc./hr./k.	2.38	1.64	1.68
Urine rate, cc./hr./k.	0.66	0.83	0.69
H_2O reabsorbed, cc./hr./k.	1.69	0.80	1.00
Urea-N reabsorbed, mgm./hr./k.	17.6	10.6	16.6
Glucose reabsorbed, mgm./hr./k.	4.5	2.1	3.4
Reabsorption per cc. of filtrate:			
H_2O (cc.)	0.72	0.47	0.53
Urea-N (mgm.)	7.76	6.04	9.75
Glucose (mgm.)	2.01	1.31	2.22

Column I. Group of 10 normal animals closest to the average plasma level of the injected animals previous to their injection.

Column II. Average of 9 collection periods from 5 animals injected with copious amounts of urea.

Column III. Group of 5 normal animals nearest to the average plasma level attained after the urea injections were made. More animals could not be used in this group because of the smaller numbers available at this relatively high urea level.

injected animals are compared on the one hand with an uninjected group selected to have a plasma level close to those prevailing before the urea injections were made, and on the other hand with a group of animals whose average plasma level compared with the experimental animals after the massive urea injections. If the kidney were "set" in its behavior by the pre-injection level, the second column of the table should correspond with the first; if the kidney behaved toward the injected urea as it does toward the endogenous urea there should be a correspondence between the second and third columns. Actually neither relation appears, but there is indication of a general depression of renal function as shown by the filtration rate, water reabsorption, urea reabsorption and glucose reabsorption.

TABLE II

Data from 110 collection periods from smooth dogfish in good physical condition which had received inulin injections but no urea, based on data of summers of 1948 and 1950

	Minimum	Average	Maximum
Urine flow, cc./hr./k.	0.11	0.63	1.72
Filtration rate, cc./hr./k.	0.53	1.82	3.51
Urea clearance, cc./hr./k.	0.05	0.24	0.81
Glucose clearance*, cc./hr./k.	0.03	0.16	0.29
Inulin U/P ratio	1.10	3.28	7.07
Urea U/P ratio	0.13	0.39	0.74
Plasma urea-N, mgm. %	730	1014	2140
Urine urea-N, mgm. %	120	373	603
Plasma glucose,* mgm. %	129	253	529
Urine glucose,* mgm. %	13	54	112
Per cent reabsorption:			
H_2O	9	66	86
Urea-N	70	87	99.5
Glucose*	86	92	99.0
Reabsorption per cc. of filtrate:			
H_2O (cc.)	0.09	0.65	0.86
Urea-N (mgm.)	5.2	8.9	18.5
Glucose (mgm.)*	1.2	2.3	3.5

* For glucose, only 51 collection periods are represented.

Since there is a paucity of data in the literature, it seems worthwhile to present some of the routine values obtained in these experiments, even though their significance for the present study is incidental. These are summarized in Table II. The values were obtained from animals which were used for a total of 110 collection periods during the summers of 1948 and 1950. There is no available explanation for the fact that the rates of urine flow obtained in a very short series of experiments in the summer of 1947 (Kempton and Steckler, 1947) were of a different order of magnitude, reaching a maximum of 3.94 cc./hr./k. as contrasted to 1.72 in the other two summers. In other respects these earlier animals were entirely in accord with the findings in 1948 and 1950, but because of the extreme differences in urine flow, and the possibility that this was a technical artifact due to unskillful handling of the animals, the few 1947 data have been omitted. Also omitted are the animals to which urea was administered, and a few periods in which

the fish appeared to be moribund. Twelve collection periods with one spiny dogfish, *Squalus acanthias*, in 1948 gave values which were entirely in line with those of the smooth dogfish.

Discussion

The data indicate that the rate of urea reabsorption is geared, directly or indirectly, to the plasma urea level. The only constant value is the average amount of urea remaining in the tubules unabsorbed from each cubic centimeter of filtrate. This relationship prevails through a range of normally occurring plasma levels in which the maximum is triple the minimum.

The reason that a similar relationship does not appear between total urea reabsorption in terms of mgm./hr./k. and the plasma concentration seems to be clear. In such a calculation the amount of urea would depend upon both the concentration in the filtrate and the volume of the filtrate. As the latter changed so would the amount of urea reabsorbed. But since the reabsorption is geared to the amount remaining unabsorbed in each unit volume of filtrate, the variations in total filtration introduce a random element which eliminates any apparent relationship.

The rate of reabsorption from each cubic centimeter of filtrate is not altered appreciably by the amount of filtrate produced nor the total amount of urea filtered. If all glomeruli are functioning all the time, differences in rate of filtration should result in a different rate of flow along the tubule, which in turn should be mirrored in variations in rate of urea reabsorption. This effect should certainly be clear when, as in these data, the rate of filtration falls along a seven-fold range. However, changes in filtration rate due to varying numbers of functional glomeruli would not result in a change of rate of flow. The fact that there is no change in urea reabsorption which can be correlated with changes in filtration rate indicates that the varying filtration rate in these experiments was produced by a change in the number of functional glomeruli rather than by increased or decreased function of individual units.

The variations in unabsorbed urea can be explained on either of two bases. These lead to two diametrically opposed conclusions in relation to the site of urea reabsorption as compared with that of water reabsorption. Variations in rate of filtration do not affect the urea reabsorption, apparently because there is no change in rate of flow of the filtrate, which has been discussed above. However, changes in amount of water reabsorption are related inversely to the amount of unabsorbed urea. This would be consistent with the idea that the osmotic effect of the greater amount of urea retained in the tubule inhibits reabsorption of water and leads to greater urine flow and a higher concentration of urea in the urine. The facts equally well fit the explanation that with less reabsorption of water there is a more rapid flow of filtrate which results in increased amounts of unabsorbed urea and higher urine levels. The first explanation would place urea reabsorption earlier in the tubule than water reabsorption or at the same level; the second would place it after the site of water reabsorption. No choice between the alternatives can be made on the basis of the present data. It is hoped that further experiments will permit a definite choice.

The reabsorption of urea cannot be isosmotic. While at lower plasma levels enough water is reabsorbed to account for an isosmotic reabsorption of all the urea

and possibly the glucose and chloride as well, at higher plasma levels only a small fraction of the urea could be absorbed as an isosmotic solution, even if all the reabsorbed water were assigned to this purpose. This therefore leaves open the possibility that the controlling factor in urea reabsorption is a constant concentration of urea remaining in the tubules, possibly through the intervention of osmotic relationships. It is perhaps more likely that we are dealing with an enzyme system which functions rapidly only when a certain concentration of urea is present in the tubules.

Summary and Conclusions

1. Water, urea and glucose reabsorption have been studied simultaneously using the inulin clearance method.

2. Variations in filtration rate appear to be due to changes in the number of functional glomeruli, rather than to changes in the rate of function of individual units.

3. Glucose reabsorption is not correlated in any way with that of urea.

4. The main factor controlling the urea reabsorption is the concentration of urea occurring normally in the plasma. Attempts to increase the reabsorption of urea by raising the plasma level result in a depression of reabsorption not only of urea but of glucose and water as well. To some extent this reduced water reabsorption is offset by a decreased filtration rate, with the result that only a very moderate diuresis ensues.

5. The percentage of the filtered urea which is reabsorbed varies with the concentration in the plasma, ranging from 70% to 99.5%, comparing quite favorably with the reabsorption of glucose.

6. On the average, the actual amount of unabsorbed urea left in each unit volume of filtrate is quite constant over a wide range of normally occurring urea levels.

7. Variations in the amount of unabsorbed urea are correlated in a presumably causal fashion with the reabsorption of water, the rate of urine flow and the concentration of urea in the urine.

8. Choice cannot be made at this time between alternate explanations of the data which would localize the site of urea reabsorption in relation to the site of water reabsorption.

9. The data show clearly that the reabsorption of urea cannot be an isosmotic one.

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THE CYTOCHEMICAL STAINING AND MEASUREMENT OF PROTEIN WITH MERCURIC BROMPHENOL BLUE[1]

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Various techniques have been adapted from protein chemistry to the cytological demonstration of proteins (*e.g.*, ninhydrin reaction, Mazia and Jaeger, 1939; Millon reaction, Pollister and Ris, 1947; Sakaguchi reaction, Thomas, 1946; Serra, 1946) but none has found wide use for both the resolution of morphological detail in terms of protein distribution and the measurement of relative protein concentrations. Danielli (1948) has presented an incisive summary of the problem and has proposed some ingenious new approaches, but these have not yet found common application.

The development of chromatographic and electrophoretic techniques which require identification of spots on filter paper has given new impetus to the study of color reactions of amino acids and proteins, and it is inevitable that the experience which is developing rapidly in this field will be carried over to the field of biological staining. Durrum (1950) devised the mercuric bromphenol blue reagent which has been widely adopted for the detection of protein spots, and the reaction has been studied in some detail by Kunkel and Tiselius (1951) and Geschwind and Li (1952). The present study deals with the application of the mercuric bromphenol blue procedure to cytological material. Its value as a simple staining technique, its capacity for bringing out in good contrast certain structures that often do not stain well by other procedures, and its specificity and applicability to the photometric estimation of proteins in cytological preparations will be considered.

Experimental

1. *Preparation of tissue.* Common cytological fixatives, such as Carnoy's, Baker's formalin, Schaudinn's and Bouin's solutions, have been employed in this study. Of all fixatives tested, only those containing osmium interfere with this reaction. The alcoholic mercury-bromphenol blue reagent (Hg-BPB) as described by Durrum would in itself have the properties of a fixative so that theoretically fixation and staining could be carried out in one step. We have found in practice that fixation of blocks of tissue proceeds too slowly in this solution, and the slow penetration sets up artifacts in protein distribution due to the transport of unprecipitated protein within the cells by the flowing fixative. However, we have found the Hg-BPB solution to be satisfactory in the fixation of cell smears and of cilia (Fig. 1). Presumably it would be adapted to other surface structures.

[1] This work has been assisted by grants from the American Cancer Society, recommended by the Committee on Growth, National Research Council, and from the University of California Cancer Research Coordinating Committee.

It is necessary to work with quite thin preparations because the staining by this method is so intense in most cases. Sections of the order of 5 microns or less are recommended.

2. *Staining.* Staining may be carried out either in the alcoholic Hg-BPB solution described by Durrum or the aqueous solution used by Kunkel and Tiselius. In both cases the solution we have used contains 10 grams of $HgCl_2$ and 100 milligrams of bromphenol blue per 100 ml. The alcoholic solution is made up in 95 per cent ethanol. The preparations are immersed in the dye solution for 15 minutes.

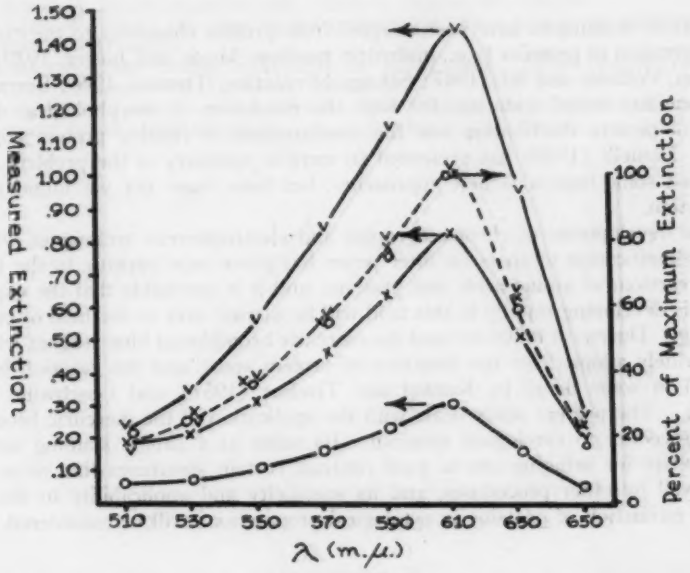


FIGURE 1. The validity of Beer's Law for the comparison of the Hg-BPB staining of objects of different optical density. Complete absorption spectra are given for the nucleolus of a sea urchin (*Strongylocentrotus purpuratus*) oocyte (—v—), cytoplasm of a rat intestinal mucosa cell (—x—) and cytoplasm of a mature sea urchin egg (—o—). The data on the egg cytoplasm are not comparable to Tables I and II since a thinner region was chosen in order to obtain a curve in a lower extinction range. In all three cases shown, a region 3 microns in diameter, producing a 3 mm. spot at the phototube level, was measured. Slit width: 2 mm.

A longer period does not increase the intensity of staining. The preparations are then washed for 20 minutes in 0.5 per cent acetic acid as described by Kunkel and Tiselius. This solution removes excess dye but does not remove the dye that is presumably bound chemically, even after 5 hours of washing. Washing in water for 15 minutes is perfectly suitable for cytological purposes, but there is no sharp end point; with prolonged treatment with water more and more dye is removed. After the acid wash, the sections are immersed in water or buffer of pH 6–7 for three minutes to convert the dye to its blue alkaline form. Treatment with ammonia

vapor, as recommended for filter paper strips, is not suitable as it produces serious distortion of morphological detail.

These three steps constitute the whole procedure and yield reproducible results. All other standard manipulations appropriate to making temporary or permanent mounts may be carried out without apparent effects on the staining of the cells, except that prolonged soaking in dilute ethanol during dehydration may lead to some loss of dye.

Results

1. *Qualitative.* A variety of materials, plant and animal, has been studied. The procedure described is believed to be of general applicability as a cytological and histological technique. In the material we have studied, there was no known structure that was not brought out in good contrast to its background by the technique, and this is not at all surprising since it is difficult to imagine the existence of a cell structure without some concomitant differentiation in protein composition and concentration. Each of the structures selected for purposes of illustration may, of course, be stained by other means, but for the most part different techniques would be required in different cases. Fixatives and stain solutions employed are designated in the legends to the photographs.

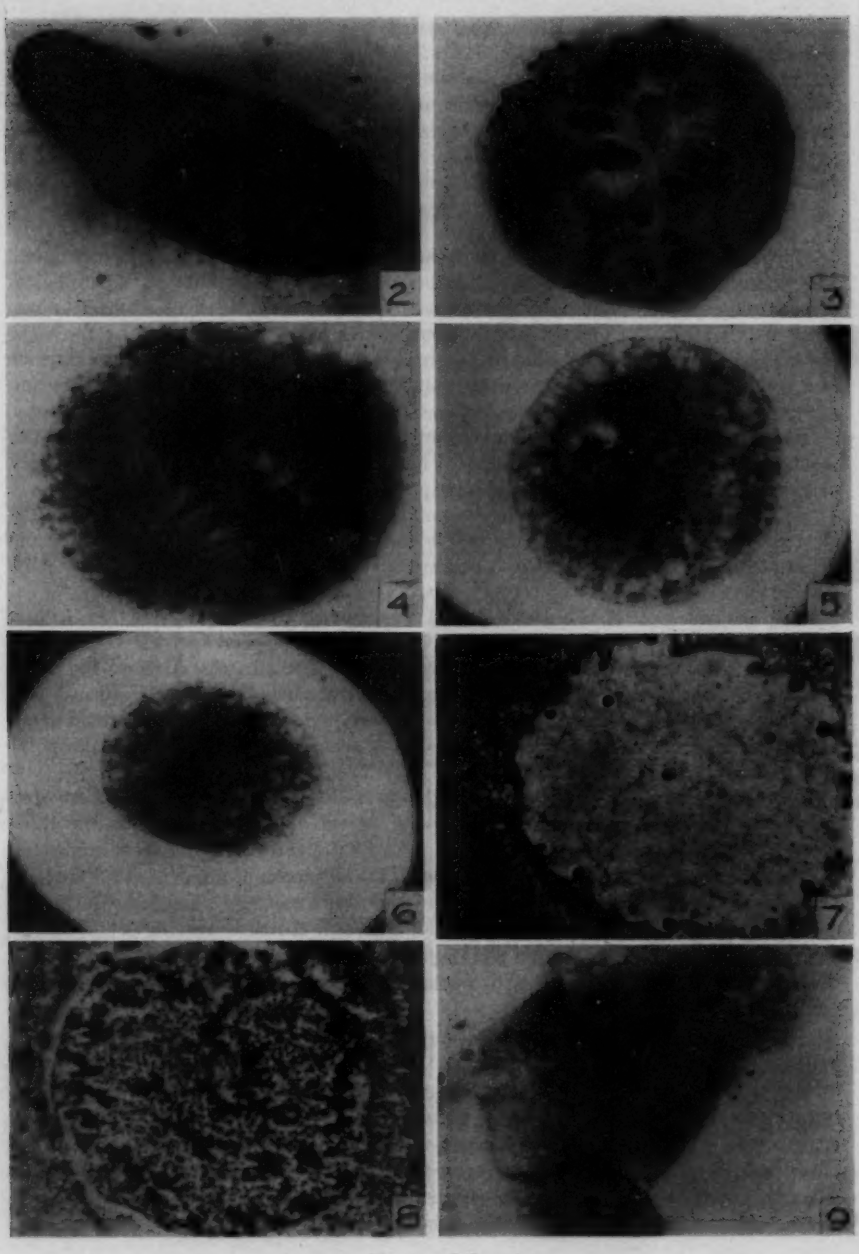
(1) *Cilia.* Figure 2 shows a *Paramecium* fixed and stained directly in the alcoholic Hg-BPB solution. This cell is too thick for the observation of internal detail but the cilia, which ordinarily require special staining techniques, appear dark and distinct.

(2) *Mitotic figures.* Figures 3 and 4 show microspore divisions in *Lilium longiflorum.* Figure 2 is a metaphase plate and is of special interest because the regions of attachment to the spindle fibers seem to be distinguished from the other regions of the chromosomes by their greater dye-binding capacity. Figure 3 is a later stage in equatorial view, showing strong staining and apparent structural detail of the spindle components. Each chromosome seems to be connected to the polar cytoplasm by a bundle of fibrils.

Figure 5 shows an early stage in the first division of an egg of *Anascaris equorum.* The material of the "achromatic figure" (by this technique the most densely staining structure in the cell) is seen in the form of two asters connected by a mass of staining material lying between the adjacent pronuclei. In Figure 6, an equatorial view of an early anaphase in the first cleavage of the same form, the complete mitotic figure, with asters, spindle and two chromosomes, is seen.

Dr. Sajiro Makino (personal communication) has applied this technique to the demonstration of meiotic figures in orthopteran and hemipteran testes with satisfactory results.

(3) *Lamp brush chromosomes.* The giant "lamp brush" chromosomes found in the oocytes of amphibian and other lower vertebrates are difficult to stain by most methods because the nucleic acid is rather dispersed. The Hg-BPB technique brings out their structure clearly. Figure 7 shows an oocyte of the frog *Rana pipiens* and Figure 8 an oocyte of the urodele *Batrachoceps attenuatus.* In the latter case, the chromosomes are more condensed and therefore more conspicuous. The frog chromosomes are highly extended, but the stain differentiates their characteristic organization as a thread with long lateral projections or loops rather well.



FIGURES 2–9.

This picture is interesting because it corresponds so well to that obtained, in more favorable species, in living nuclei (Duryee, 1950).

(4) *Amoeba.* In our experience, the nucleus of *Amoeba proteus* is brought out only in very poor contrast with nucleic acid stains. The concentration of nucleic acids seems to be relatively low in this large nucleus. Figure 9 shows an individual *Amoeba proteus* stained by the Hg-BPB technique, showing the nucleus to be a structure where the protein concentration is much higher than that obtaining in the cytoplasm.

These preparations, as well as others we have obtained, contain a number of features of considerable cytological interest, which will not be discussed at this time. Some will be evident from inspection of the figures. The crispness and clarity of the results have been noteworthy if only because they are obtained by a rapid method that requires no modification from one type of material to another.

2. *Quantitative.* Preliminary to a study of the specificity of the Hg-BPB stain, a series of measurements has been made to determine whether the Beer and Lambert laws apply to material stained by this method. The apparatus and technique were those previously described by Alfert (1950), except that a Beckmann Model B spectrophotometer was used as a light source.

The absorption maximum of the dye-protein complex in tissue is 610 mμ. The test of the applicability of Beer's Law was carried out in two ways. First, extinction measurements at various wave-lengths were made on three different regions within the same cell: the cytoplasm, nucleus and nucleolus of oocytes of the sea urchin *Strongylocentrotus purpuratus.* In Table I the absolute and relative extinction values are given for four wave-lengths. The measurements given in the table were made on the same cell, cut at 5 microns. Therefore the extinction values for different regions are comparable. The "relative" values were calculated by letting the measured extinctions at 610 mμ equal 100. It is seen that while the intensity of staining varies from one region to another, the relative extinctions are nearly the same at different wave-lengths, as required if Beer's Law is valid for the situation. Figure 1 leads to the same conclusion by a somewhat different approach. Complete absorption curves were taken for three materials—

FIGURES 2–9.

FIGURE 2. *Paramecium caudatum.* Fixed and stained in aqueous Hg-BPB. Whole mount. × 330.

FIGURE 3. *Lilium longiflorum.* Microspore division. Polar view of metaphase plate. Fixation: Carnoy's solution. Sectioned at 8 μ. Stain: aqueous Hg-BPB. × 1240.

FIGURE 4. *Lilium longiflorum.* Microspore division. Equatorial view of anaphase. Fixation, sectioning, and staining as in Figure 3. × 1330.

FIGURE 5. Egg of *Anascaris equorum.* Stage of close approach of pronuclei. Fixation: Carnoy's solution. Section at 8 μ. Stain: aqueous Hg-BPB. × 970.

FIGURE 6. Egg of *Anascaris equorum.* Equatorial view, first cleavage division. Note staining of asters, spindle, and the two chromosomes. Prepared as in Figure 5. × 830.

FIGURE 7. Ovary of *Rana pipiens.* "Germinal vesicle" of oocyte. Note staining of nucleoli and fine "lamp brush" chromosomes. Fixation: Bouin's solution. Sectioned at 10 μ. Stain: aqueous Hg-BPB. × 300.

FIGURE 8. Ovary of *Batrachoceps attenuatus.* "Germinal vesicle." "Lamp brush" chromosomes seen in longitudinal and transverse section. Note structure of projecting loops. Fixation: Bouin's solution. Sectioned at 10 μ. × 350.

FIGURE 9. Part of an *Amoeba proteus,* showing intense protein-staining of nucleus. Fixation: Carnoy's solution. Squash preparation. Stain: aqueous Hg-BPB. × 430.

TABLE I

The validity of Beer's Law for the Hg-BPB staining of cell contents (sea urchin oocyte)

Wave-length	Extinction					
	Cytoplasm		Nucleus ("sap")		Nucleolus	
	Measured	Relative	Measured	Relative	Measured	Relative
530	0.187	33	0.194	36	0.265	27
570	0.314	55	0.314	59	0.606	61
610	0.568	100	0.537	100	1.00	100
650	0.068	12	0.089	17	0.052	15

the sea urchin oocyte nucleolus, the cytoplasm of mucosa cells of rat intestine, and the cytoplasm of the mature sea urchin egg. The extinction values at 610 mμ range from 0.3 to 1.44. The absolute extinction curves are evidently parallel, but the close parallelism is best shown in curve 4, where the relative extinctions for all three materials are plotted on the same scale by setting the maximum value as 100. Over such a wide range of extinction values, deviations from Beer's Law through recognized mechanisms (Sandell, 1944) would be expressed as changes in the absorption curves.

A rather elegant means of testing the applicability of Lambert's Law was provided by sectioned sea urchin egg populations, where we frequently encountered cases in which sections of two cells slipped and partially overlapped. It was thus possible to compare the absorption by single sections of two eggs with the absorption of the regions where these were superimposed. The results are given in Table II where it is seen that the extinction values are additive to a satisfactory degree. The several tests listed in Table II were made on different pairs of cell-sections. Regions 4 microns in diameter were measured, giving a 4 mm. spot at the level of the phototube.

In visual inspections of slides stained by this method, it often appeared that the most intensely stained preparations also seemed to have a more reddish hue. At

TABLE II

Tests of the validity of Lambert's Law for measurement of Hg-BPB staining of sea urchin egg cytoplasm. Measurements made on adjacent 5 micron sections (a and b) and on region where they overlap (a + b)

Test No.	Extinction at 610 mμ			
	Section a	Section b	Measured a + b	Deviation from calculated a + b
1	0.602	0.613	1.202	−1%
2	0.612	0.777	1.284	−8%
3	0.614	0.665	1.362	+6.5%
4	0.754	0.599	1.237	−9%
5	0.612	0.615	1.236	+1%

first this seemed to suggest the complication of metachromasia or some related effect. However, the photometric measurements revealed no difference between the bluer and the redder preparations. The reddish appearance of densely stained material may be explained in terms of the spectral sensitivity of the human eye, but is irrelevant to the present discussion since it has no influence on objective measurements.

The demonstration of the validity of photometric laws does not in itself imply that we are measuring the concentration of any normal constituents of the cell. In discussing this common misconception in relation to Beer's Law, Sandell (1944, p. 61) says, ". . . this law simply states that the extinction must be proportional to the concentration of the colored substance; it does not state that the extinction must be proportional to the analytical concentration of the constituent which forms the colored substance." Such an assurance can only come from chemical investigation of the staining reaction, as described below. But the above data do indicate that, insofar as we can interpret the dye-binding reaction, the microphotometric measurements will accurately follow its stoichiometry.

Since the Millon reaction has proved to be useful in photometric studies on cell proteins, it is of interest to compare it with the Hg-BPB reaction. A disadvantage of the Millon technique is that the compound formed absorbs so weakly in the visible. While satisfactory extinctions may be obtained in the near-ultraviolet

TABLE III

The binding of Hg-BPB by various substances on filter paper

Substance	Moles dye bound per mg.
Starch	0
Glycogen	0
DNA	5.9×10^{-9}
Cholesterol	5.4×10^{-9}
Bovine serum albumin (cryst.)	1.20×10^{-6}
Chymotrypsin (cryst.)	1.06×10^{-6}
Pepsin (cryst.)	1.03×10^{-6}
Histone (pH 4)	1.40×10^{-6}

region, the structures to be measured are difficult to locate visually. We have compared the extinction values of sea urchin egg cytoplasm stained by the Millon procedure (measured at 355 mμ) and by the BPB procedure (measured at 610 mμ). The average of 10 measurements by the BPB procedure for a given series of sections was 0.646. The Millon reaction gave an average extinction of 0.121. Thus the BPB reaction provides greater optical density with a given concentration of material as well as an absorption maximum in a more convenient range.

3. *Specificity and quantitative significance.* Few tests for protein actually measure the mass of protein. What is usually determined is some chemical group that is characteristic of proteins. The tests give information concerning the amount of protein present only insofar as one has independent information as to the distribution of the particular group or groups in the protein under observation.

Obviously, the first consideration is the qualitative specificity of the BPB reaction; whether the dye is combined by non-protein constituents of the cell. We have tested, on filter paper, the dye-binding by a series of substances that might confuse the reaction. The following table (Table III) indicates that the dye-

binding by the non-protein materials tested is negligible compared with that of protein. The procedure was exactly as described above, carried out on filter-paper spots. The amount of dye was estimated after extraction of the paper with ammoniacal acetone, the standard being a known solution of dye in the same solvent.

The question of the BPB reaction of amino acids and polypeptides has been studied in detail by Geschwind and Li (1952). From their work it appears that many free amino acids and peptides would bind dye, but the dye complexes—with the exception of those involving histidine and possibly cysteine—would be dissolved away during the washing of the preparation.

From the cytological standpoint, one is most interested in the possibility of distinguishing protein in structures containing nucleic acid. We therefore studied the BPB reaction in cells from which nucleic acids had been removed by extraction with hot 5 per cent trichloroacetic acid for 15 minutes. No decrease in the BPB staining is observed. In fact, certain structures, especially the chromosomes, now stained more intensely, as would be anticipated if the nucleic acid were blocking potential dye-binding groups. The fact that the intensity of chromosome staining increased is consistent with the view that many of the basic groups of nuclear proteins are bound to the acid groups of nucleic acid.

Kunkel and Tiselius (1951) have shown that the amount of dye bound is proportional to the amount of protein over a wide range. They found, however, a 30 per cent difference in the dye-binding per milligram of albumin and gamma globulin. We have tested a number of pure proteins and mixtures containing proteins (tissue homogenates) on filter paper and have consistently observed the same linearity and variability. Our highest and lowest values for dye bound per milligram protein differ by about 40 per cent. Our procedure differed from that of Kunkel and Tiselius only in that the dye was extracted from the paper by ammoniacal acetone. On the average, one milligram of protein combined about 10^{-6} moles of dye. Taking 120 as a representative weight of an amino acid residue, this would mean that one molecule of dye is bound for, approximately, every 10 amino acid residues present.

In view of this variability the method will be applicable to the exact comparison of total protein concentrations only where the qualitative differences are expected to be small. It will be suitable for the approximation of large differences, since the variation of dye-binding among various proteins studied is by no means one of order of magnitude.

The variability is true of most protein methods, which generally involve the measurement of certain characteristic groups in proteins. It does not seem likely, from the results reported, that the method is specific for a single group. As mentioned, we have found that in a variety of proteins the ratio of moles of dye bound to amino acid residues present is roughly 0.1. Examining the composition of the proteins, we find no single type of amino acid that accounts for 10 per cent of those present.

If we examine those groups which could potentially enter the reaction, these seem to be the NH_2 groups of basic proteins, which might combine by direct salt formation, and the SH groups, aromatic groups and free COOH group which might be coupled to the dye through the mercury.

The experiments showing enhanced staining of chromosomes after extractions

with trichloroacetic acid indicate that the basic groups of basic proteins may contribute to the dye-binding. This would be a simple case of acid-staining, in the microscopists' terminology, and would not be expected to involve combination with mercury. A number of experiments, both with pure proteins on filter paper and with tissues, suggests that this direct combination of the dye with basic groups is, in fact, largely limited to basic proteins, but that a mechanism involving coupling through Hg is responsible for the major part of the staining of tissues and of ordinary proteins. In the work with pure proteins on filter paper it was shown that spots of protamine stained equally well whether the Hg was present or absent. On the other hand, ovalbumin stained only in the presence of Hg; when Hg was omitted, there was a loose attachment of dye which readily washed out in the standard procedure. Since the function of the Hg might merely be that of keeping the protein on the paper, we returned the albumin-containing papers, which had been washed free of dye after treatment with the Hg-free reagent, to the normal Hg-containing dye solution. The spots now stained normally and the dye could not be washed out. Therefore the Hg plays a role in coupling the dye to the protein. In these experiments, the protein spots were fixed with 3:1 alcohol-acetic acid and dehydrated with alcohol.

Exactly the same relationship held for tissues. When these were treated with a dye solution without Hg, they seemed to take up a considerable amount of dye, but most of it was removed on washing. Under the microscope, a typical picture of acid-staining was observed. It is suggested that a comparison of staining with and without Hg, and before and after the extraction of nucleic acids, might provide a basis for estimating the relative amount of basic protein and the extent to which the basic groups were free, but this has not been investigated in detail.

The groups which might combine the dye through Hg, the SH, aromatic and free COOH groups, might be differentiated by blocking with appropriate group-reagents, and it is proposed to investigate this possibility.

Discussion

For the cytochemist who is interested in estimating the total concentration of protein, rather than some characteristic side chain, the ideal method would be one in which each amino acid or peptide linkage would produce an equivalent light absorption. Such a method would be provided by the biuret reaction, for instance, though the extreme alkaline conditions required render this unsuitable for cytological purposes. The next best approximation is a method which will register such a variety of groups as to minimize, by a statistical "averaging-out," the consequences of compositional differences among proteins. The Hg-BPB method would seem to have this advantage. Inspection of tables of protein composition shows that the aggregate percentage of the amino acids potentially reacting in this method varies less from protein to protein than the percentage of any one amino acid.

The method has the further advantages that there is no class of proteins that will not be stained significantly and that, insofar as the composition of the test-object is known, the quantitative differences in the dye-binding by different proteins may be assessed accurately by parallel studies on filter paper. The studies that have already been made by Kunkel and Tiselius and by ourselves show that proteins and protein mixtures may readily be analyzed by simple filter-paper techniques with

excellent correlation between the amount of protein present and the amount of dye bound. The variation that we have discussed previously seems to be a genuine index of qualitative differences among proteins, and subject to analysis.

The fact that the dye is combined to different groups and by at least two different mechanisms offers certain interesting possibilities. For instance, the staining without Hg appears to be a typical case of acid staining, which has been used by others (*e.g.*, Schrader and Leuchtenberger, 1950) as a measure of basic proteins. If bromphenol blue is used, and the staining in the presence and absence of Hg is compared, one has a system of comparison of basic protein to total protein in which at least the photometric units (extinction, optical density) can be compared directly in terms of the number of dye-binding groups. This would not be the case if different dyes were used for the two classes of proteins. When it becomes possible to sort out the various groups that combine the dye through Hg, the same advantage will obtain. In this way, the Hg-BPB method should be a useful complement to those methods which are selective for one group, such as the Millon method for phenolic residues and the methods specific for SH groups (Tahmisian and Brues, 1951; Bennett, 1951).

Summary

1. The mercuric bromphenol blue reaction as used for development of protein spots on filter paper has been found to be applicable to the cytological staining of proteins.

2. The optimum procedure is identical in detail with that described by Kunkel and Tiselius for filter paper spots, except that a neutral aqueous solution is substituteed for ammonia vapor in the final color development.

3. The sharp and intense staining of protein permits good differentiation of structures often difficult to observe, such as cilia, spindle elements, regions of spindle fiber attachment to chromosomes and "lamp brush" chromosomes.

4. The procedure is specific for proteins and those peptides which are not removed in the washing procedure.

5. The preparations stained by this procedure follow the Beer and Lambert Laws in microspectrophotometric measurements. The absorption maximum is at 610 millimicrons.

6. Basic proteins bind the dye under the conditions of the method even when Hg is omitted. Other proteins bind the dye by coupling through Hg. As expected, structures containing basic proteins show enhanced staining after removal of the nucleic acid.

7. The number of groups in various proteins binding the dye in this procedure varies somewhat, but the average is about one dye-binding group per 10 amino acid residues.

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STUDIES IN THE DEVELOPMENT OF FROG HYBRIDS. IV. COMPETENCE OF GASTRULA ECTODERM IN ANDROGENETIC HYBRIDS

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Hybrid embryos obtained from the fertilization of *Rana pipiens* Schreber ova with sperm of *Rana sylvatica* Le Conte develop in a seemingly normal manner to the beginning of gastrulation (Moore, 1946). In the majority of cases the hybrids form a tiny dorsal lip and differentiate no further. They remain as arrested gastrulae for nearly a week and then cytolize. The failure of further differentiation of these hybrids has been found to be correlated with reduced competence of the presumptive epidermis (Moore, 1947) and reduced inductive ability of the dorsal lip (Moore, 1948) as compared with normal embryos.

In a further effort to dissociate the factors responsible for the cessation of development in the hybrids, a study has been made of androgenetic hybrids of *R. pipiens* ♀ × *R. sylvatica* ♂ obtained by removal of the maternal chromosomes. The development of these haploid hybrids tests the ability of the *sylvatica* nucleus to influence the development of the *pipiens* egg devoid of *pipiens* chromosomes. It must not be imagined, however, that the enucleated *pipiens* ovum is merely a passive substrate for a foreign sperm to mould. We shall be studying the effect of *sylvatica* genes on the egg cytoplasm that was organized in the ovary under the influence of *pipiens* genes. There is a considerable body of evidence to indicate that the development of the egg up to late blastula or early gastrula is already determined at the time it leaves the female (Moore, 1941). In the case of these androgenetic hybrids, therefore, we should not expect an effect of the *sylvatica* chromosomes during the cleavage and blastula stages.

Materials and Methods

Eggs were obtained from a *R. pipiens* by pituitary injections. Some were fertilized with *pipiens* sperm and others with *sylvatica* sperm. Using the method of Porter (1939), the maternal chromosomes were removed from a number of the eggs. The four classes of embryos obtained were normal *pipiens*, *pip* × *pip*; haploid *pipiens*, (*pip*) × *pip*; hybrids, *pip* × *syl*; and haploid hybrids, (*pip*) × *syl*.

The *pip* × *pip* embryos developed normally. The (*pip*) × *pip* embryos were identical with the diploid normals during the early stages. The first deviation from normality occurred during gastrulation, when there was a slight retardation in the rate of development. In later stages morphological abnormalities, characteristic of the haploid syndrome (Porter, 1939; Moore, 1950), appeared and the embryos died as edematous larvae.

The *pip* × *syl* hybrids developed normally to the beginning of gastrulation. Thereafter, development was completely abnormal (Moore, 1946). Wrinkles and

furrows formed in the blastocoel roof, which later became smooth as the entire embryo swelled. Eventually the blastocoel roof appeared to collapse and the embryo was left as a much wrinkled gastrula. Cytolysis began when the *pip* × *pip* embryos were in stage 20 (gill circulation) or 21 (cornea transparent).

The (*pip*) × *syl* embryos developed normally until the late blastula stage, as has been noted previously by Ting (1951). Epiboly was not as extensive as in *pip* × *syl* and the dorsal lip never formed. A grayish region on one side of the embryo may have been an indication of where the dorsal lip would have appeared, if differentiation had proceeded further. The (*pip*) × *syl* embryos, like the diploid hybrids, became swollen and remained alive until the normal *pipiens* controls reached stage 21. Thus, at a temperature of 20°, they remained as arrested blastulae for approximately one week. During this period the haploid hybrids appeared perfectly healthy and did not exhibit the characteristic wrinkles, pits and furrows of the diploid hybrids, nor did they shrink in size before cytolysis.

Since diploid hybrids of *pip* × *syl* have a dorsal lip, and haploid hybrids of (*pip*) × *syl* do not, it is possible to be sure of using the desired embryos for transplantation. The few embryos in the (*pip*) × *syl* group which formed a dorsal lip were assumed to be diploid, due to failure of removal of the maternal chromosomes, and were not used in the transplantation experiments. The (*pip*) × *pip* early gastrulae can be definitely distinguished from the *pip* × *pip* embryos by the slight retardation in development.

Experiments

Competence of the gastrula ectoderm was tested by transplanting pieces of the blastocoel roof of an early gastrula to the pronephric region of older embryos, in a manner previously described (Moore, 1947). Such transplants, under the influence of the host cells, form neural tissue and other structures if competent, as Holtfreter (1933) has demonstrated. The host in all of the experiments was *Rana palustris* Le Conte. Embryos of this species are much lighter in color than *R. pipiens* embryos and, therefore, host and donor cells are readily distinguishable. This difference in pigmentation is apparent in histological preparations as well. The hosts were in stage 17 (tail-bud) at the time of transplantation. One donor contributed transplants to two hosts.

Fourteen transplants of (*pip*) × *syl* presumptive ectoderm were made and compared with similar transplants of 8 (*pip*) × *pip*, 10 *pip* × *syl* and 2 *pip* × *pip*. The (*pip*) × *pip* transplants served as the controls for haploidy, while the *pip* × *syl* experiments formed the chief basis of comparison. The behavior of *pip* × *syl* and *pip* × *pip* tissue has been studied extensively in previous experiments (Moore, 1947), so relatively few operations of this material were performed for the present experiments.

It was found that when the control *pip* × *pip* were early gastrulae, the (*pip*) × *pip* were slightly earlier gastrulae; the *pip* × *syl* showed pigment at the dorsal lip and no invagination; and the (*pip*) × *syl* were late blastulae. Since it was desirable to have the different transplants in the same relative stage of differentiation, transplants were made from *pip* × *pip*, (*pip*) × *pip* and *pip* × *syl* when they were in stage 10 (early gastrulae). Since the *pip* × *syl* are arrested at stage 10, and the formation of the dorsal lip is retarded, they were used when they had been

in this stage for a short time. Control *pip* × *pip*, fertilized at the same time and kept under the same conditions, were in stage 11 (semi-circular blastopore). The (*pip*) × *syl* had to be used in stage 9 (late blastulae), as they never form a dorsal lip. Since their development was retarded at this time, they were used when the control *pip* × *pip* were in stage 12 (yolk plug).

In the transplants from the three control types, the *palustris* hosts were allowed to develop to stage 21. They were then fixed and studied in serial section. In the transplants involving (*pip*) × *syl* tissue, the hosts were fixed in stage 20, since some of the transplants were unhealthy at that stage.

Results

The 14 transplants of (*pip*) × *syl* ectoderm showed no differentiation whatsoever. The cells remained as large, late blastula cells. There was little variation among the 14 cases and Figures 1 and 2 may be taken as typical of the results. It was noticed that the donor cells stained much less intensely than the host cells with fast green.

The 10 *pip* × *syl* transplants showed slightly better differentiation than the haploid hybrid transplants. The cells were smaller in size than in the (*pip*) × *syl* experiments. A two-layered epidermis was formed with an underlying mass of tissue which could not be called either neural or neuroid. The degree of differentiation was similar to that shown in Moore (1947), Figure 8. This represents the lowest degree of differentiation that can be expected with this tissue.

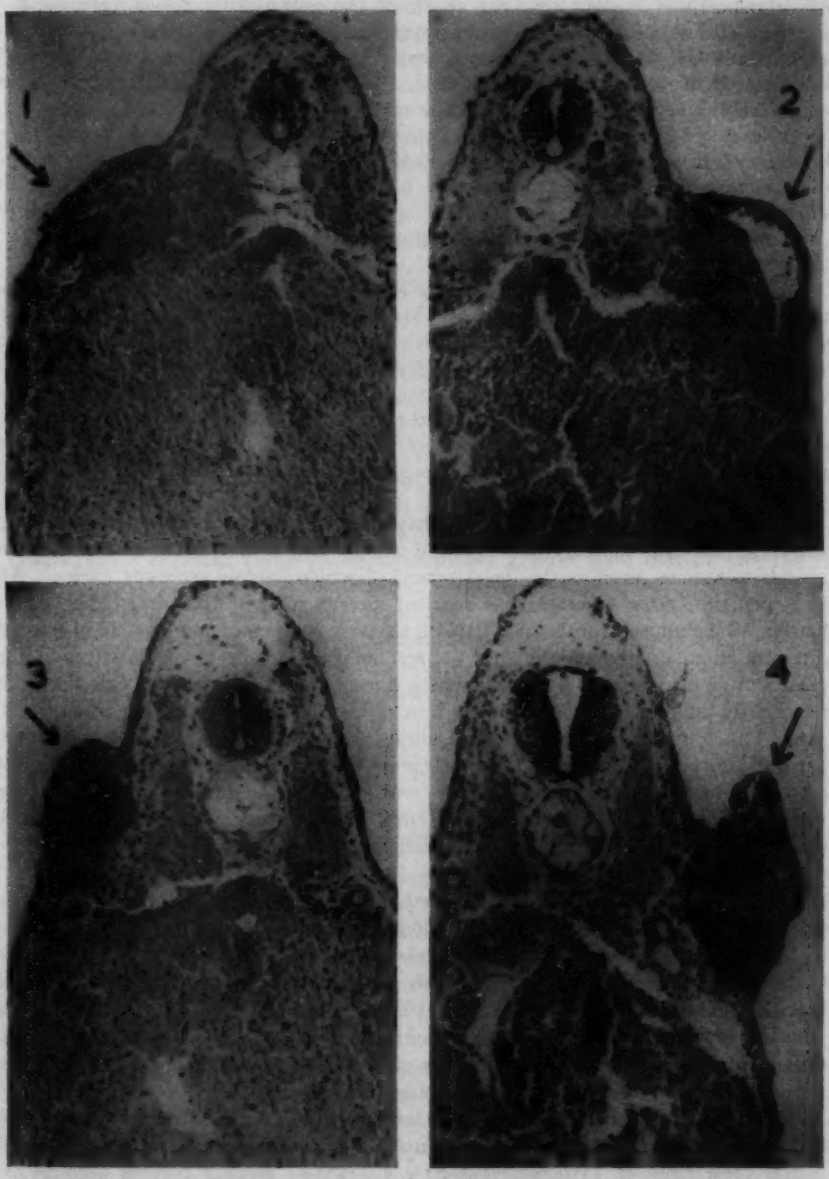
The 8 (*pip*) × *pip* transplants were distinctly better in their differentiation than either previously mentioned class. The transplants formed a two-layered epidermis with underlying neuroid masses. Figures 3 and 4 are typical.

The two *pip* × *pip* transplants showed the greatest differentiation. As in accordance with earlier work (Moore, 1947), neural tissue was formed.

Discussion

From the data described above, it is evident that the presumptive ectoderm of (*pip*) × *syl* is lacking in competence and, when transplanted, cannot differentiate any further than in the entire haploid hybrid embryo. This result throws some light on the factors involved in the cessation of development of the diploid hybrids. We may imagine that there are three main components in a *pipiens* ♀ × *sylvatica* ♂ hybrid, namely: *pipiens* cytoplasm, a haploid set of *pipiens* chromosomes and a haploid set of *sylvatica* chromosomes. When all three components are present, normal development ceases at the beginning of gastrulation. Since the combination of *pipiens* cytoplasm plus a haploid set of *pipiens* chromosomes alone can produce an embryo which reaches the larval stage of development, it is clear that the addition of a haploid set of *sylvatica* chromosomes is having an antagonistic effect on the functioning of the *pipiens* chromosomes, or of the *pipiens* cytoplasm or of both. Since the development of an embryo with *pipiens* cytoplasm and a haploid set of *sylvatica* chromosomes is even less after the removal of the haploid set of *pipiens* chromosomes, it is evident that there is an antagonistic effect between the *sylvatica* chromosomes and the *pipiens* cytoplasm. That this is a real antagonism has been demonstrated by the transplantation experiments, where no response can be elicited

PLATE I



FIGURES 1 AND 2. Development of androgenetic *pipiens* ♀ × *sylvatica* ♂ gastrula ectoderm in *Rana palustris*. The region of the donor tissue is indicated by an arrow.

FIGURES 3 AND 4. Development of androgenetic *pipiens* gastrula ectoderm in *Rana palustris*. The region of the donor tissue is indicated by an arrow.

in the haploid hybrid tissue, even though in a normal environment. From these experiments it is evident that the result of the antagonistic action between the *sylvatica* chromosomes and the *pipiens* cytoplasm is actually lessened by the presence of a set of *pipiens* chromosomes, since the development of the diploid hybrid is greater than that of the haploid hybrid, and transplanted ectoderm is capable of considerably greater differentiation (Moore, 1947).

A finding of considerable interest is the evidence of a gradation in competence of the presumptive ectoderm of the various types of embryos studied. The results indicate that diploid *pipiens* presumptive ectoderm is more competent than haploid *pipiens* presumptive ectoderm. The last is more competent than the diploid hybrid presumptive ectoderm which is more competent than the haploid hybrid presumptive ectoderm.

Although only two transplants of *pip* × *pip* tissue and 8 of (*pip*) × *pip* tissue were made, in a previous experiment (Moore, 1947) 18 transplants of *pip* × *pip* gastrula ectoderm were described. In each one of these 20 transplants, neural tissue was formed from the donor cells, whereas none of the 8 (*pip*) × *pip* transplants formed good neural tissue. Their response was at a distinctly lower level of differentiation which is customarily called neuroid. More recent experiments (unpublished data) have shown that out of 33 *pip* × *pip* transplants, 91% formed neuroid or neural tissue, but such a response was shown by only 44% of 32 (*pip*) × *pip* transplants.

Similar testing of the competence of haploid tissue has not been studied by other workers. In all probability the degree of competence of such tissue will vary from species to species, even as the development of whole haploid embryos varies. An androgenetic *Triton taeniatus* has reached metamorphosis (Baltzer, 1922; Fankhauser, 1938) and obviously such haploid tissue is competent and capable of normal differentiation. Although the (*pip*) × *pip* embryos die as young larvae and the presumptive ectoderm has been shown to have reduced competence, this does not necessarily indicate that such tissue is incapable of good differentiation when transplanted to a normal diploid host. Dalton (1946) has shown that transplanted haploid neural crest cells of *Triturus rivularis* can produce the normal pigment pattern, whereas the whole merogone reaches only the tailbud stage. Hadorn (1932, 1937) has shown that haploid *Triton palmatus* tissue is capable of normal, though retarded, differentiation, in contrast to the whole merogones, which die between tailbud and forelimb stage.

The diploid hybrid, *pip* × *syl*, showed reduced competence of the presumptive ectoderm when compared with the diploid and haploid *pipiens* embryos. Histological differentiation of *pip* × *syl* tissue is poor, even when incorporated with the host neural tissue (Moore, 1947). This is in marked contrast to transplants of lethal diploid tissue described by Lüthi (1938). He found that androgenetic *Triton palmatus* ♀ × *Salamandra maculosa* ♂ gastrula tissue would develop normally when transplanted into a diploid *T. palmatus* gastrula. The whole hybrid embryo develops no further than middle gastrula stage (Schönmann, 1938).

The complete lack of differentiation of the haploid hybrid tissue, (*pip*) × *syl*, is also in marked contrast to studies made by other workers. Haploid hybrid neural crest cells of *Triturus rivularis* ♀ × *Triturus torosus* ♂ have been trans-

planted by Dalton (1946) and were found capable of producing a pigment pattern, whereas the whole merogone developed only to the tailbud stage. De Roche (1937) found that transplanted presumptive ectoderm of androgenetic hybrid tissue between *Triton alpestris* ♀ × *Triton palmatus* ♂ developed normally, whereas whole merogones only developed as far as closed neural folds. Since these haploid hybrids differentiate at least as far as neurulae, their developmental capabilities should be greater than (*pip*) × *syl* tissue, which does not develop to a gastrula stage. Moreover, the diploid hybrids of these species reach metamorphosis, in contrast to the *pip* × *syl*, which die as early gastrulae.

Hadorn (1932) found that implanted androgenetic hybrid gastrula tissue of *Triton palmatus* ♀ × *Triton cristatus* ♂ formed normal tissue, except for pycnotic head mesenchyme. The whole merogones reached closed neural folds, with pycnotic head mesenchyme. In later experiments in which chimeras of androgenetic *T. palmatus* ♀ × *T. cristatus* ♂ gastrulae and diploid hybrid *T. palmatus* ♀ × *T. cristatus* ♂ gastrulae were studied, Hadorn (1937) found that the androgenetic hybrid tissue was incapable of differentiating anterior head structures. Additional experiments with the androgenetic hybrid material indicated that the ectoderm is incapable of forming an optic vesicle and that there may be reduced inductive ability of the head organizer. These latter experiments, which show a localized lack of competence, affecting anterior head structures, of (*palmatus*) × *cristatus* tissue, are of particular interest when compared with our experiments, which show no competence of (*pip*) × *syl* tissue. We would expect the developmental capabilities of (*palmatus*) × *cristatus* tissue to be considerably greater than that of (*pip*) × *syl* tissue, since diploid hybrids of *palmatus* × *cristatus* may metamorphose and the haploid hybrids reach closed neural folds.

From this comparison of similar transplantation experiments of other workers with ours, it is evident that the degree of differentiation of such transplants varies with the species used. When a comparison of transplants of diploid, haploid, diploid hybrid and haploid hybrid tissues, such as were described in this paper, is made, however, it is found that there is a gradation in the differentiating capabilities of such tissues, from a maximum for the diploid to none for the haploid hybrid tissue.

Summary

1. In an effort to dissociate the factors responsible for the failure of development in *Rana pipiens* ♀ × *Rana sylvatica* ♂ hybrids, a study has been made of the development of haploid embryos composed of *pipiens* cytoplasm and *sylvatica* chromosomes. These androgenetic hybrids develop only to the late blastula stage.

2. The competence of the presumptive ectoderm of these haploid hybrids was tested by transplantation to neurulae of *Rana palustris*. The results indicated a total lack of competence.

3. Aside from the main problem with which the paper is concerned, it was found that the various classes of embryos used could be arranged in order of decreasing competence of the presumptive ectoderm. The sequence was as follows: diploid *pipiens*, haploid *pipiens*, *pipiens* ♀ × *sylvatica* ♂ diploid hybrids and (*pipiens* ♀) × *sylvatica* ♂ haploid hybrids.

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COMPOSITION OF THE SWIMBLADDER GAS IN DEEP SEA FISHES[1]

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In an earlier study of the composition of the swimbladder gas in bottom-dwelling marine fishes caught at depths down to 220 meters, it was found that at all depths the partial pressure of nitrogen in the swimbladder was usually close to that of the surrounding sea water, *i.e.*, 0.8 of an atmosphere (Scholander, Claff, Teng and Walters, 1951). CO_2 was present only in traces, and thus the pressure build-up above 0.8 atmosphere seemed to be due to oxygen only. A slight but definite increase in the nitrogen pressure with depth was apparent from the data, but this was considered at least partly to be an artifact due to changes in the gas composition after the catch.

Non-bottom-dwelling species were often found to have a partial pressure of nitrogen in their bladders far above 0.8 of an atmosphere. These fishes were in buoyancy equilibrium at a very shallow depth, and hence might have acquired their high nitrogen values when equilibrating at much lesser depths than that at which they were caught, *i.e.*, as a consequence of vertical migrations.

In the summer of 1952 we had the opportunity to extend the survey down to a depth of some 950 meters, *i.e.*, about four to five times deeper than before, with the result that now a fuller and more accurate picture of the performance of the gas gland in relation to depth can be presented.

Material

Material for the present investigation was obtained at the Lerner Marine Laboratory, Bimini, Bahamas, December 24, 1951, to January 2, 1952, and during two trawling cruises from the Woods Hole Oceanographic Institution. The latter were conducted off the New England coast by the dragger *Cap'n Bill II* from July 10 to 17 and 23 to 30, 1952. Some 260 specimens were analyzed, covering 29 species of fish caught between 120 and 950 meters' depth.

At the Lerner Marine Laboratory the fishes were obtained at depths of 300–420 meters. They were caught at the bottom on a baited hook fastened to a heavily weighted wire line and were pulled to the surface within 3–5 minutes by means of a motor driven reel. The wire passed over a meter wheel which indicated the depth. Gas samples from the following species were analyzed:

Epinephelus	Grouper
Epinephelus mystacinus	Black grouper
Lutianus vivanus	Long-fin red snapper

[1] Contribution Number 632 from the Woods Hole Oceanographic Institution.

Fishing on the *Cap'n Bill* was done by means of an otter trawl, dragging the bottom for 30 minutes. The time of ascent of the net from the bottom to the surface may be estimated at about 30 minutes for the greatest depths. The depth of the stations was continuously indicated on a fathometer. Gas samples from the following fishes were taken and analyzed on board:

		Known depth range in meters
Alepocephalus sp.		890
Antimora viola	Blue hake	560–1830
Argentina silus	Herring smelt	60–670
Coryphaenoides rupestris	Round-nose ratfish	700–2200
Cottunculus thomsonii	Deep sea sculpin	190–1570
Dicrolene sp.		840–890
Macrourus bairdii	Common ratfish	140–2300
Macrourus berglax	Smooth-spined ratfish	180–1240
Merluccius bilinearis	Silver hake	0–870
Notacanthus phasganorus		870
Peristedion miniatum	Deep sea robin	170–350
Sebastes marinus	Rosefish	30–1680
Simenchelys parasiticus	Slime eel	360–1620
Synaphobranchus pinnatus	Long-nosed eel	240–2660
Tautogolabrus adspersus	Cunner	0–130
Urophycis chesteri	Long-finned hake	60–990
Urophycis chuss	Squirrel hake (ling)	0–540
Urophycis regius	Spotted hake	160
Urophycis tenuis	White hake (ling)	0–540

The data on the depth range are taken from Bigelow and Welsh (1924) and from Goode and Bean (1895), or are from our own records.

Methods and Reliability of Data

The micro-gas-analytical method used gives the carbon dioxide and oxygen percentages through absorption with KOH and alkaline pyrogallol[2] respectively (Scholander *et al.*, 1951). The accuracy is about ± 0.2 to ± 0.3 per cent of the true value. The unabsorbed fraction ("nitrogen") of the gas sample, in addition to nitrogen, may contain noble gases and organic gases.

The amount of organic gases was determined by combustion in a separate chamber and subsequent analysis for CO_2 in the one-half cc. analyzer (Scholander, 1947). The plungers of the syringes with which the samples for these analyses were taken had been lubricated with concentrated lithium chloride solution, and care was taken throughout to avoid organic contaminations. The results are given in Table I and show that the amount of CO_2 formed by combustion of the swimbladder gas is usually no more than a few hundredths of one per cent. Hence organic gases play no significant role in the build-up of the gas pressure in the swimbladder of deep sea marine fishes. The traces of organic gases present in the samples may have been derived from bacterial activity in the gut of the fish.

[2] In the described technique the oxygen is absorbed by running a large excess of fresh pyrogallol down over the gas bubble. This procedure keeps carbon monoxide formation down to sub-detectable values, even in samples of pure oxygen, and agrees with results obtained by Kilday (1950), who used essentially the same absorption principles in a Haldane-type macro-method.

No carbon monoxide could be detected in gas samples from *Urophycis chesteri* and *Macrourus berglax*. Other species were not tested.

Schloesing and Richard (1896) analyzed the swimbladder gas of *Muraena helena* caught at 88 meters' depth and found that the argon over nitrogen-plus-argon ratio was 1.85%. Similarly in *Synaphobranchus pinnatus*, caught at 1385 meters' depth, the ratio was 1.94%. With a ratio in air of 1.18%, this means about a 60% increase in the argon over nitrogen-plus-argon ratio in these fishes. The samples were small and the analyses were not considered very accurate, although probably significant as to the increase mentioned. We have as yet no data on the content of argon in our material.

In our discussion of the correlation of the gas composition with depth we have assumed (a) that the fish are in a steady state of equilibrium at the depth of the catch, *i.e.*, that they have remained at this depth for a substantial period of time, and (b) that this gas composition has not changed materially since the time the fish was caught.

TABLE I

Carbon dioxide content of swimbladder gas before and after combustion

Species	Depth of catch (m.)	% N_2	% CO_2		ΔCO_2
			Before Combustion (a)	After Combustion (b)	(b)-(a)
Antimora viola	820	7.8	1.31	1.32	0.01
	820	7.2	0.84	0.90	0.06
Coryphaenoides rupestris	720	10.0	0.18	0.19	0.01
	720	11.6	0.67	0.72	0.05
	720	—	0.85	0.96	0.11
Macrourus bairdii	820	8.2	0.03	0.16	0.13
	610	8.2	0.65	0.67	0.02
Sebastes marinus	440	11.2	0.37	0.38	0.01
	440	—	0.34	0.41	0.07
Synaphobranchus pinnatus	820	6.2	1.96	1.98	0.02
Urophycis tenuis	610	7.8	0.37	0.40	0.03
	610	5.8	0.20	0.20	0.00
	610	6.2	0.18	0.19	0.01

As to the first assumption, we know that the Bimini fishes were at the bottom when they were caught, and Bigelow and Welsh (1924) consider most of our northern deep sea species as decidedly bottom-dwelling species. To what extent these fishes might migrate up and down along the bottom slope is not known.

As to the second assumption, there is good evidence that the changes in gas composition that take place from the time the fish is caught until the time the gas

sample is secured are so small that they could not change the conclusions significantly. Thus, the Bimini fishes, which were secured between five and ten times faster than the northern fishes, gave results which superimposed on the northern data. Two sets of experiments showed that changes in the gas composition after the catch occur only slowly. Bloated and more or less moribund individuals of eight species of our deep sea fishes, left on deck, in or out of water, showed an increase of at most one per cent in the nitrogen percentage in 30 minutes. In two species of fishes from much shallower depths, as reported earlier, the increase was larger, but still insignificant when compared with the total nitrogen.

In other experiments oxygen was injected into the peritoneal cavity of two remoras (*Echeneis naucrates*) and one puffer (*Spheroides maculatus*), caught in shallow water. The amount injected was about ten times the amount of swimbladder gas normally present in fishes of similar weights. In these fully alive fish the nitrogen per cent of the injected gas increased in half an hour by 1.4 and 0.4%, respectively. A fish at 1000 meters' depth contains 100 times as much gas as a surface fish, and if such an amount could have been injected into these remoras or the puffer the percentage nitrogen increase would have been entirely negligible.

Also, simple calculation shows that even if the entire amount of nitrogen dissolved in the tissues of a fish were transferred into the swimbladder, which was then expanded 100 times, the increase in the nitrogen would amount to only a few tenths of a per cent. Finally, the amount of oxygen present in a swimbladder at 100 atmospheres' depth is so great that respiratory oxygen removal could not materially change the nitrogen percentage either. We feel, therefore, that our data must reflect essentially the conditions which existed in the swimbladder when the fish was caught.

Composition of the Swimbladder Gas in Relation to Depth

In Figure 1 a composite diagram is presented of the previous (Scholander *et al.*, 1951) and present data, extending from the surface forms to the deep sea forms at 950 meters. The diagonal straight line represents a nitrogen percentage which corresponds to a partial pressure of nitrogen of 0.8 atmosphere, *i.e.*, that of the surrounding sea water. We assume here that nitrogen values to the right of this line are due to something other than diffusion equilibrium and that the balance of the pressure is due to oxygen secretion. It is clear from the figure that the nitrogen values found at greater depths deviate increasingly from the line showing the atmospheric nitrogen tension.

Figure 2 shows the partial pressures of the nitrogen and oxygen as calculated from the percentages found and from the depths at which the fish were taken. In fishes living near the surface the nitrogen and oxygen tensions were generally found to be close to those in air, indicating that here the diffusion exchange between the swimbladder and the outside water dominates the picture. From the surface value of 0.8 atmosphere the nitrogen pressure increases in an over-all linear fashion with depth, often attaining values as much as 10 to 15 atmospheres higher than the nitrogen tension in the surrounding sea water.

Much of the considerable spread in the data in Figure 2 is due to specific differences among the fishes. In Figure 3 we have plotted the nitrogen for four species of fish, covering a wide range of depth. It is indicated that the increase in

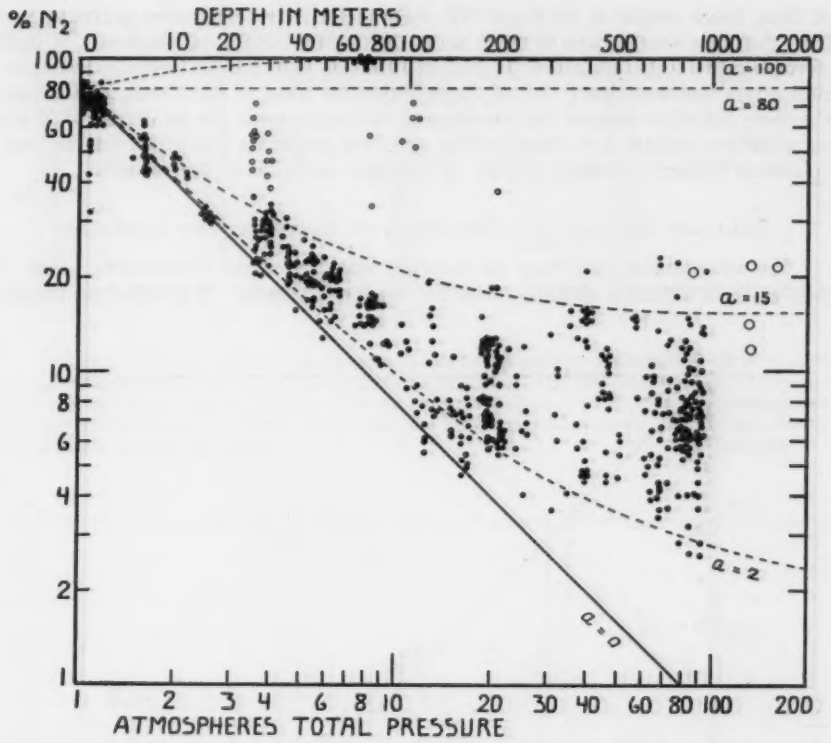

FIGURE 1. Composite graph of the nitrogen concentration found in swimbladder gas from fishes at various depths. The diagonal full-drawn line represents the percentage of nitrogen which at any given depth would give a partial pressure of nitrogen of 0.8 atmosphere. Both the present and previous data (Scholander *et al.*, 1951), as well as Hüfner's data (1892) for *Coregonus* (near 100% N_2), are given by solid circles. The large open circles are data on *Simenchelys parasiticus* (1674 m.) and *Synaphobranchus pinnatus*, given by Schloesing and Richard (1896). The small open circles are data from scup and silver hake, where the high nitrogen most likely is due to vertical migration. The dotted lines represent percentages of the total gas pressure due to nitrogen corresponding to values for *a* of equation (1). The empirical data indicate that *a* in our material lies usually between 2 and 15.

nitrogen tension with depth in each species is more or less linear, the curves for the different species having individual slopes. These differences are also apparent in Figures 4 and 5.

Sebastes is a fish that apparently stays at the bottom in the daytime, but moves off the bottom at night. This could tend to produce a nitrogen value higher than that corresponding to the bottom depth (Scholander *et al.*, 1951). However, high nitrogen is also found in species like the deep sea sculpin, *Cottunculus*, and many others which are most likely always in close proximity to or on the bottom. Many

of these fishes caught at depths of 900–1000 meters have a nitrogen percentage so high that they would have to move up to 100 meters' depth, or shallower, if their nitrogen were to be explained by simple diffusion from the sea water. Such vertical migrations are clearly out of the question for most of our fishes, and we are therefore forced to assume that nitrogen as well as oxygen can be deposited in the swimbladder against very considerable pressure gradients. This, of course, corroborates Hüfner's findings (1892) of nitrogen secretion in the whitefish.

Relation Between Gas Deposition by Diffusion and Secretion

The swimbladder gas may be resolved into two main components. One is derived from diffusion exchange with the outside sea water. The other constitutes

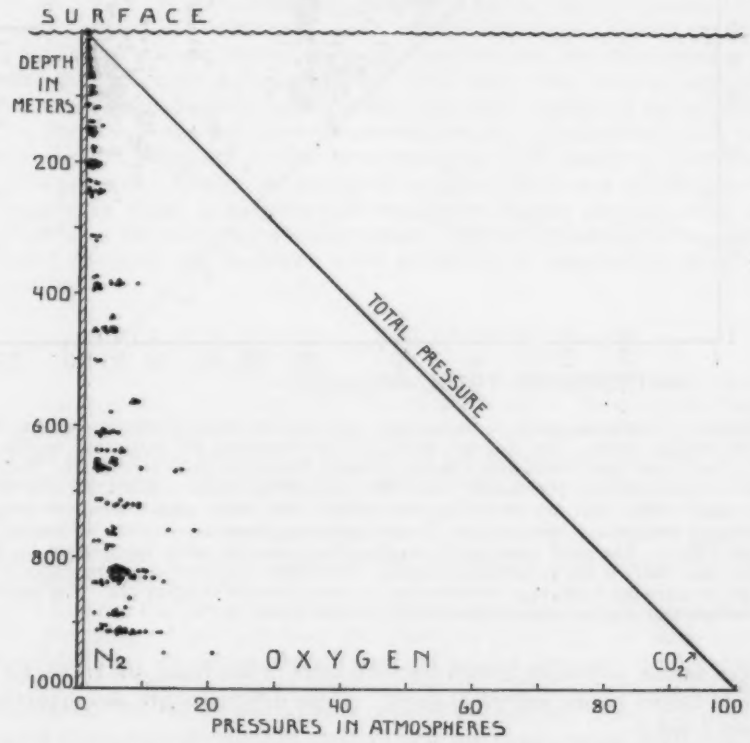

Figure 2. The partial pressures of nitrogen, oxygen and CO_2 calculated from the percentages and the depths. The shaded area is equal to the partial pressure of nitrogen in the sea water. The partial pressure of nitrogen in the swimbladder is to the left of each point, of oxygen to the right. The partial pressure of the CO_2 is usually less than what is represented by the thickness of the drawn diagonal line. The over-all picture suggests a linear increase in the partial pressure of the nitrogen with depth.

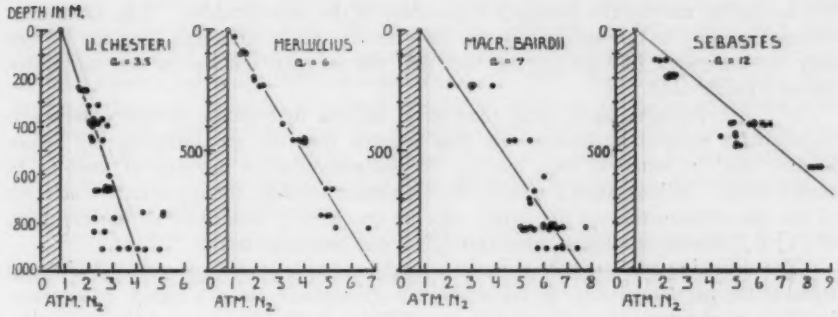

FIGURE 3. Partial pressures of nitrogen in the swimbladders of *Urophycis chesteri*, *Merluccius bilinearis*, *Macrourus bairdii*, and *Sebastes marinus*. The data suggest that the nitrogen in excess of 0.8 atmosphere increases linearly with depth from a value of zero at the surface. The different slopes of the lines indicate that the proportions of nitrogen and oxygen secreted are different in different species.

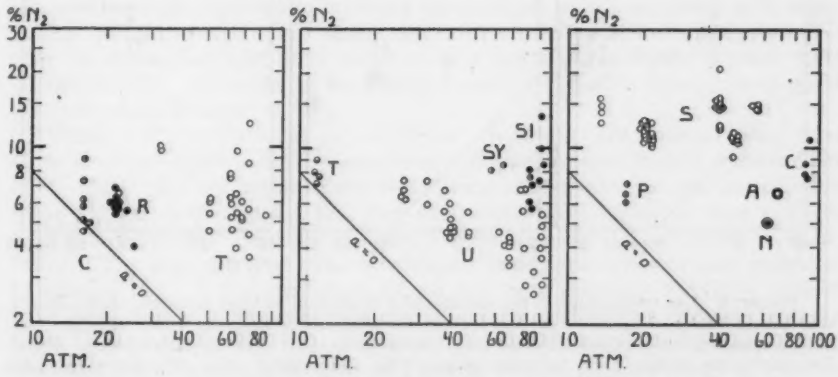

FIGURE 4. Gas composition in the swimbladder in relation to total pressure. Left: *Urophycis chuss* (C), *Urophycis regius* (R), *Urophycis tenuis* (T). Middle: *Tautogolabrus adspersus* (T), *Urophycis chesteri* (U), *Simenchelys parasiticus* (SI), *Synaphobranchus pinnatus* (SY). Right: *Peristedion miniatum* (P), *Sebastes marinus* (S), *Cottunculus thompsonii* (C), *Notacanthus phasganorus* (N), *Argentina silus* (A). These fishes were taken off the New England coast. The N_2 percentages of *Simenchelys* and *Synaphobranchus* obtained by Schloesing and Richard (1896) are entered on Figure 1. They are about twice as great as those shown from our data on these species.

what may be called the secreted [3] part which is responsible for the pressure build-up. As the nitrogen tension is known to be constant and very near to 0.8 atmosphere at all depths (Rakestraw and Emmel, 1938; Hamm and Thompson, 1941), we shall

[3] We shall use the word "secretion" to mean any process whereby gases are deposited into the swimbladder so as to increase potentially or really the partial pressure above that in the ambient medium.

discuss below mainly the nitrogen deposition in the swimbladder. The same considerations may be applied also to the oxygen deposition, although here the picture may be somewhat blurred by the fact that the oxygen tension in the sea water varies considerably.

If a fish remains for a long time at a definite hydrostatic pressure where it maintains a neutral buoyancy, one may assume that the gas entering the swimbladder will be equal to that leaving the swimbladder in amount as well as in composition. Where such a steady-state situation obtains, the quantitative relation of the gas composition to the depth may be empirically described as the resultant of (1) a diffusion exchange term and (2) a gas secretion term.

The diffusion exchange component would lead to a partial pressure of nitrogen equal to the partial pressure in sea water, 0.8 atmosphere, times a factor, F, expressing the completeness of equilibrium between the swimbladder gas and the sea water, *i.e.*, $0.8 \times F$.

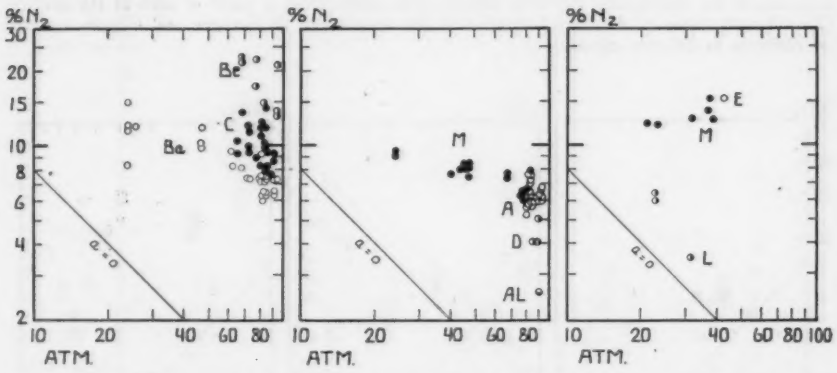

FIGURE 5. Gas composition in the swimbladder in relation to total pressure. Left: *Macrourus bairdii* (BA), *Macrourus berglax* (BE), *Coryphaenoides rupestris* (C). Middle: *Merluccius bilinearis* (M), *Antimora viola* (A), *Dicrolene* sp. (D), *Alepocephalus* (AL). Right: *Epinephelus mystacinus* (M), *Lutianus vivanus* (L), *Epinephelus* (E). The last three fishes were taken off Bimini. All the others were caught off the New England coast.

The gas secretion component represents an additional quantity of nitrogen which has entered the swimbladder as the result of secretion and which increases the partial pressure by an amount represented by $\frac{a}{100} \times D$, where a is the percentage of nitrogen present in the swimbladder as the result of secretion and D is the depth in atmospheres.

The total partial pressure, P_{N_2}, is thus given by

$$P_{N_2} = (0.8 \times F) + \left(\frac{a}{100} \times D\right) \tag{1}$$

The factor F is introduced into the diffusion term for the following reason. Near the surface D is small and in most fishes the gas diffusion exchange with the

sea water is so complete that a near-air composition is maintained in the swimbladder. This does not hold for all species, however. Even when kept in a shallow tank for a long time tautogs usually have much less than 80% N_2 in their swimbladders (Safford, 1940; Scholander *et al.*, 1951, Fig. 7). This fish, then, seems to have an exceptionally well developed isolation of its swimbladder with respect to diffusion, and this may be expressed by a factor F which is smaller than one. Although difficult to verify, it seems likely that deep sea fishes would have developed a high degree of isolation of their swimbladders against diffusion (low F) as a means for reducing gas loss.

We may now scrutinize somewhat further the idea that the nitrogen deposited into the swimbladder to develop a pressure $\left(\frac{a}{100} D\right)$ is a more or less constant fraction of the oxygen pressure $\left(\frac{100 - a}{100} D\right)$. In Figure 1 we have plotted curves for a series of a values (0, 2, 15, 80 and 100), according to equation (1). It will be seen that curves calculated using the a values of 2 and 15 describe fairly well the upper and lower limits of nitrogen admixture in the deposited swimbladder gas of our marine fishes. A fish like *Coregonus* which deposits near to 100% nitrogen would be expected to present a nitrogen percentage increasing with depth. A fish with an a value of 80 would maintain this percentage at all depths. We have no explanation why there seems to be a gap in the a values between some 20 and 90–100. As indicated by Figure 3 and by Hüfner's data, a seems to be a species characteristic.

Hüfner (1892) found that six out of nine whitefish (*Coregonus acronius*) from 60 to 80 meters' depth in the Bodensee had a nitrogen content in their swimbladders of 99–100%. At the surface these fishes were bloated with enough gas to make them buoyant at the bottom, and they must hence have deposited their nitrogen against a hydrostatic pressure of some 6–8 atmospheres (*cf.*, Scholander *et al.*, 1951). The data have been entered in Figure 1 and agree with the ideas embodied in equation (1). It is a pleasure to record that these seemingly incredible findings have lately been fully corroborated and extended by observations on fishes from the Great Lakes made by Mr. R. L. Saunders from the laboratory of Dr. F. E. J. Fry, Department of Zoology, University of Toronto.

Composition of the Gas Secreted

We have so far discussed the gas composition as it is found in the swimbladder at various depths, which leads us to believe that deep sea fishes deposit in their swimbladders not pure oxygen, but oxygen together with a fraction, a, of inert gases ("nitrogen"), a being high in some species and low in others. We shall now consider to what extent the a values might reveal what is the composition of the gas that has actually been secreted.

The gas pressure in the swimbladder in excess of the one atmosphere due to the combined pressure of nitrogen + oxygen in the sea water is the resultant of (1) the gas secretion into the bladder and (2) the diffusion losses through its wall. The swimbladder in deep sea fishes is capable of maintaining with low gas loss a pressure gradient of one hundred or more atmospheres through the thin air bladder

wall, and it is therefore clear that the exit mechanism for the gases constitutes a potent diffusion barrier. It seems likely that the secreted gases at sub-maximal depths are produced with a pressure substantially higher than the ambient hydrostatic pressure. Only if the diffusion coefficients for the different gases were the same could the gas composition in the swimbladder equal the composition of the secreted gas. The diffusion coefficient for nitrogen through a variety of animal tissues has been found to be only around one half of that of oxygen (Krogh, 1919). The steady state requires that the molar amount of each gas leaving the bladder through diffusion loss should equal the molar amount secreted. A steady state can therefore occur only when the swimbladder has backed up enough partial pressure for each gas component to allow it to pass the diffusion barrier in an amount equal to that secreted. The pressures of nitrogen and oxygen accumulated in the swimbladder above the ambient partial pressures were empirically found to be $\frac{a}{100} \times D$ atmospheres and $\frac{100 - a}{100} \times D$ atmospheres respectively (equation (1)). The diffusion losses, and therefore secretion, of these gases would hence be $\frac{a}{100} \times D \times n$ and $\frac{100 - a}{100} \times D \times o$, where n and o are the diffusion coefficients for nitrogen and oxygen respectively. Recalculated on a per cent basis, the secreted nitrogen percentage, N_{2_s}, would relate to a according to

$$N_{2_s} = \frac{100\,a}{\frac{o}{n}(100 - a) + a}. \qquad (2)$$

With the ratio of $o/n = 2/1$, the secreted N_2 percentage would be $\frac{100\,a}{200 - a}$. This means that for low values of a, like the ones found, it might be anticipated that the secreted mixture would have a *nitrogen* percentage equal to about $\frac{1}{2}\,a$. Conversely, if a were near 100, as found in *Coregonus*, the *oxygen* percentage of the secreted gas would be about twice as high as the oxygen percentage found. Thus, if the nitrogen percentage in the swimbladder of a fish at 1000 meters' depth is found to be 4.8%, then a is 4.0% and the secreted mixture is likely to be 2% N_2 and 98% O_2. If in a *Coregonus* the a value is 95, the secreted gas is likely to be 90% N_2 and 10% oxygen.

Schloesing and Richard (1896) found the argon fraction in the swimbladder "nitrogen" to be some 60% higher than in air. From the solubility of argon in water and its molecular weight one would expect argon to have twice as high a diffusion coefficient through animal tissue as nitrogen. If, therefore, argon and nitrogen entered the swimbladder in the same ratio as in air one would expect a lower argon fraction in the swimbladder rather than a higher. Evidently, therefore, these two gases enter the swimbladder in proportions different from those in air. We are totally in the dark as to how the presumably inert argon and nitrogen have attained the high pressures found in the swimbladder.

We wish to express our gratitude to Dr. Alfred C. Redfield for his stimulating

interest. We were fortunate to have all possible cooperation from Dr. William C. Schroeder, who conducted the deep sea cruises off the New England coast. He identified our specimens and supplied us with information on the depth ranges of the fishes. The Captain and the crew of the dragger *Cap'n Bill II* did everything to facilitate our work on board.

We are indebted to Dr. C. M. Breder, Jr., at the American Museum of Natural History, for arranging our stay at the Lerner Marine Laboratory. We wish to extend our thanks to Mr. Michael Lerner for his generous cooperation in securing deep sea material at Bimini and to Mr. Marshall Bishop for providing us with the most excellent facilities in the laboratory. We wish to thank Mr. Vladimir Walters, of New York University, for his excellent assistance, and we are much obliged to Mrs. Susan I. Scholander for help in the field and in the preparation of the final manuscript.

Summary

1. The composition of the swimbladder gas has been determined in 26 species (260 specimens) of marine deep-sea fishes taken at known depths between 200 and 950 meters.

2. The partial pressure of nitrogen in the bladder steadily increases with depth until it reaches some 5–15 atmospheres at a depth of 900 meters, revealing that not only oxygen but also nitrogen is transported into the swimbladder against a considerable gradient. This corroborates the findings of Hüfner (1892) that the whitefish (*Coregonus*) is able to secrete pure nitrogen against a hydrostatic pressure of 6–8 atmospheres.

3. The data, together with previous observations from shallower depths, show that the nitrogen tension at all depths may be expressed by (1) a diffusion term of 0.8 atmosphere, plus (2) a secretion term which, although different for different species of fish, is in each species a constant percentage of the total secreted gas pressure. Among our fish the secretion term ranges from about 2% to 15% nitrogen. In the whitefish it is apparently 100%. In a steady-state situation the percentage nitrogen actually secreted into the swimbladder is probably lower than the nitrogen percentage indicated by the secretion term because oxygen is more easily lost by diffusion through animal tissue than is nitrogen.

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BODY TEMPERATURES OF WHITE-FOOTED MICE IN RELATION TO ENVIRONMENTAL TEMPERATURE AND HEAT AND COLD STRESS[1]

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Many mammals are completely homoiothermic only with a relatively narrow range of external temperature, particularly some of the more primitive mammals which, with respect to thermoregulatory ability, are intermediate between the true poikilotherms and the more highly evolved mammals (Britton and Atkinson, 1938; Kredl, 1928; Martin, 1930; Morrison, 1945, 1946; Sutherland, 1897; Wislocki and Enders, 1935). Body temperatures of more advanced mammals are influenced by changes in air temperature but do not undergo the wide fluctuations that are characteristic of primitive mammals (Gaalaas, 1945; Seath and Miller, 1946). Among mammals the bats seem to be distinct in that, except during periods of activity, they appear to be essentially poikilothermic (Hock, 1951; Reeder and Cowles, 1951).

As a group the rodents show varying degrees of perfection of thermoregulation. The most labile temperatures are generally found among the hibernating species, although some non-hibernators also have inconstant body temperatures (Morrison and Ryser, 1951; Wade, 1930; Wislocki, 1933). The term "heterotherm" has been applied to certain rodents and other mammals which show rather wide fluctuations in body temperatures in relation to the ambient temperature as contrasted with the more perfectly regulating homoiotherms.

Various investigators have attempted to relate body temperatures of rats and mice to air temperatures and have pointed out differences due to both air temperature and sex (Bierens de Haan, 1922; Hart, 1951a, 1951b; Herrington, 1940; Morrison and Ryser, 1951; Przibam, 1917; Sumner, 1913).

Early studies on the effect of previous acclimation at high and low temperatures on the thermoregulatory ability of rodents and other mammals are few (Gelineo, 1934, 1940; Schwabe, Emery and Griffith, 1938). Only within recent years has this problem been studied intensively (Adolph, 1950, 1951; Adolph and Lawrow, 1951; Scholander *et al.*, 1950; and others). However, many of the mechanisms involved in acclimation to heat and cold still remain obscure.

Methods and Materials

The mice used in this study were northern white-footed mice, *Peromyscus leucopus noveboracensis* (Fischer), which were trapped from wild populations in the vicinity of Champaign-Urbana, Illinois, during 1947 to 1949. All individuals were acclimated in the laboratory at temperatures from 20–24° C. for periods of 20–30 days before any body temperature measurements were made.

[1] Contribution from the Zoological Laboratory, University of Illinois, Urbana. The author wishes to express appreciation to Dr. S. C. Kendeigh for his sympathetic guidance in carrying out this study.

The temperature chambers used in these experiments have been described in a previous paper (Sealander, 1951a).

Deep body temperatures were measured with a portable indicator potentiometer using fine copper-contantan thermocouples. The usual practice was to insert the thermocouple into the animal's esophagus, since it was presumed that possible damage to internal tissues might be less and because less difficulty was experienced in securing temperatures in this way. Thermocouples were periodically checked for accuracy against a standard thermometer.

As activity exerts an appreciable influence on body temperature (Britton and Kline, 1939; Bullough, 1949; Hart, 1951a; Kendeigh, 1945), a sufficient number of records was usually obtained to secure both maximum and minimum temperatures. A series of temperatures was taken at about one-minute intervals until no further changes in temperature could be detected for several minutes. Temperatures were recorded while the animal was held in the left hand or confined in a small cone of wire netting. The animals were handled with a glove to minimize any changes in body temperature due to radiation or conduction of heat from the handler. The mice struggled briefly when the thermocouple was inserted and then became quiet. If they continued to struggle no additional readings were taken. Body temperatures were almost invariably highest during the first minute or two of recording and then underwent only minor fluctuations during the remaining period of measurement. After the initial rise, body temperatures continued to decline gradually and usually from 8–12 minutes elapsed before they remained constant. Body temperatures of mice subjected to low temperatures were recorded within about a ten-minute period to preclude any rise in temperature from exposure to warmer room temperatures. Body temperature readings followed the same general pattern in these mice.

Automatically recorded subcutaneous temperatures of mice at different air temperatures were obtained with thermocouples inserted under the skin (Sealander, 1951b) which were connected to a strip-chart potentiometer that recorded the balance point of the circuit every three minutes.

Experimental Results

Behavior responses. Reactions of individual white-footed mice to temperature stress were, in general, quite similar to those which have been frequently reported for laboratory rats, mice, hamsters and guinea pigs.

Initial exposures at moderately low temperatures ($-10°$ to $-15°$ C.) were accompanied by periods of gnawing at the cage alternating with vigorous grooming movements. Motor activity was evidenced by frequent exploratory trips to different corners of the cage. Lowering the temperature to about $-20°$ C. intensified these behavior responses for a short while, but gnawing and motor activity soon became infrequent although grooming movements persisted. At air temperatures between $-20°$ and $-30°$ C. there were only occasional outbursts of motor activity in the form of running.

Erection of the fur and reduction of the amount of exposed surface by curling into a ball became more prominent as the mice became quiet. Frequent turning movements were made, apparently in an effort to achieve a more compact body surface. At very low temperatures ($-25°$ to $-35°$ C.) the feet, ears and tail

quite often became frost-bitten due to greater exposure and contact with cold surfaces.

Polypnea and shivering were characteristic responses to cold. Their onset varied with individual mice and with the suddenness of exposure to low temperatures. Rapid breathing and shivering movements were most prominent at very low temperatures but appeared rather abruptly in mice that were suddenly placed at temperatures of about − 20° C. Respiration became weak and shallow and shivering disappeared in hypothermal mice. Loss of equilibrium appeared with the approach of the lower lethal body temperature, and the lethal point was often marked by a final weak burst of activity followed by a total loss of balance and culminated in a clonic-tonic convulsion of the type described by Frings and Frings (1952).

At temperatures of 25° C. and above the mice decreased their motor activity and generally assumed sleepy, relaxed postures. When air temperature was around 35° C. the extremities became noticeably suffused with blood. At 40° C. motor activity was usually quite pronounced during the first half-hour of exposure but then ceased almost entirely. The mice salivated quite freely at this temperature and often assumed a partially erect posture with the forepaws resting upon the sides of the cage. In the neighborhood of 50° C. crawling movements were evident and the belly fur became streaked and matted with saliva as the mice crawled over the bottom of the cage.

Death occurred very rapidly in the range of temperature between 40° and 50° C. but was not accompanied by the convulsive seizures and anoxic symptoms noted at low temperatures.

Minimal recoverable body temperatures. The extremes of low temperature which many species of mammals can endure for a short period of time and still survive are often very great. For example, the laboratory rat is able to maintain a constant body temperature for several hours at − 30° C. (Giaja and Gelineo, 1933). Many species of arctic mammals are notably resistant to cold as pointed out by Scholander and his associates (1950).

A number of experiments have been performed (Barbour *et al.*, 1943, 1944; Britton, 1922; Britton and Atkinson, 1938; Haterius and Maison, 1948; Luyet and Gehenio, 1940) in which mammals were subjected to very low temperatures and underwent recovery. In the majority of mammals body temperatures from about 14° to 20° C. were the lowest which could be tolerated for any length of time with complete spontaneous recovery at room temperatures, but bats and ground squirrels tolerated body temperatures from a few tenths to about two degrees below 0° C. Other mammals, such as hamsters (Adolph, 1951; Adolph and Lawrow, 1951), tolerate body temperatures as low as 4° C. for varying lengths of time.

Very few minimal recoverable body temperatures have been determined for different species of mice. Sumner (1913) exposed white mice to air temperatures of − 2° to 2° C. for about five hours and noted complete recovery of two mice from temperatures of 20.6° and 12.5° C. after exposure to an air temperature of 28.5° C. Kendeigh (1945) reported the recovery of a white-footed mouse, *Peromyscus maniculatus gracilis*, after its body temperature had fallen to 23.4° C. after several hours exposure in a trap.

In the present study no systematic attempt was made to determine minimal recoverable body temperatures but a few incidental observations were obtained.

Two male white-footed mice exposed to air temperatures from − 12° to − 23° C. had body temperatures of 10° and 23° C., with exposures of 65 and 80 minutes, respectively, when removed to room temperatures of 26–27° C. Both underwent complete spontaneous recovery. Another male exposed to − 20° C. for 70 minutes underwent partial recovery from a body temperature of 6° C. when removed to a room temperature of 27° C. but subsequently died. These few records indicate that this species probably falls within the general range of tolerance for non-hibernating rodents of the same general size class. There was little or no activity by the mice during the time they were exposed to the low temperature.

Lethal temperatures. Upper and lower air temperature limits between which mammals can remain completely homoiothermal over long periods of time are but incompletely known for many species. In general, these limits vary with the animal's total heat production and the degree of body insulation.

TABLE I

Esophageal temperatures of nineteen adult Peromyscus leucopus noveboracensis at room temperatures of 25° to 28° C.

Females				Males			
Number of records	Temperature in ° C.			Number of records	Temperature in ° C.		
	Minimum	Maximum	Mean		Minimum	Maximum	Mean
1	—	38.7	—	10	35.8	39.2	37.0
8	35.5	39.0	38.3	1	—	38.2	—
1	—	39.5	—	9	37.0	40.5	38.2
9	37.5	39.6	37.9	11	36.0	38.7	37.3
8	37.3	39.8	38.5	9	35.5	39.3	37.4
10	36.8	40.5	38.3	8	38.7	39.5	38.9
10	37.2	39.7	38.1	10	36.0	39.0	37.5
12	36.3	38.7	37.7	12	35.7	37.8	37.0
110	35.7	40.2	38.2	12	36.1	37.6	37.3
				108	35.4	38.3	37.7
Mean	36.6	39.5	38.1		36.2	38.8	37.6

Rodents are known to be much more susceptible to high temperatures than many other mammals. The usual explanation for this greater susceptibility is their lack of sweat glands, coupled with a high respiratory rate that cannot be increased greatly for cooling.

A series of esophageal temperatures taken on 15 adult *Peromyscus leucopus,* of both sexes, at the moment of death as indicated by the last convulsive respiratory movement, gives some indication of the lower lethal body temperature for this species. The mice were subjected to temperatures ranging from − 12° to − 35° C. while deprived of food and water. Lower lethal body temperatures ranged from 3.5° to 5.0° C. with a mean of 4.6° ± .5° C. In general, the duration of exposure before death occurred showed a progressive increase (45 minutes to 8.5 hours) with rise in temperature. However, there was no evident correlation between the lower lethal body temperature and either air temperature or length of exposure. Inas-

much as body temperature changes rapidly after thermogenic ability is once lost it is difficult to fix an absolute lethal temperature. Heat production is probably permanently impaired in deer mice at body temperatures in the neighborhood of 10° C. as evidenced by lack of permanent recovery from temperatures in this vicinity.

A few experiments were performed in which deer mice were subjected to falling or gradually lowered air temperatures. In all cases the body temperature underwent the most rapid decline when the ambient temperature was around − 12° to − 15° C. All of the animals concerned in the experiments were freshly caught summer animals. Although it would appear that this is a critical zone of temperature for summer animals it seems probable that the critical zone might be lower for animals previously acclimated at low environmental temperatures.

TABLE II

Comparison of esophageal and subcutaneous temperatures of seven adult Peromyscus leucopus noveboracensis at 25° C. when under anaesthesia and when fully recovered***

Sex	Body Temperature (° C.)			
	Under light anaesthesia at point of recovery		Two hours after recovery from anaesthesia	
	Mean esophageal temperature	Mean subcutaneous temperature	Mean esophageal temperature	Mean subcutaneous temperature
F.	37.1(3)	35.9(3)	38.4(4)	37.2(4)
M.	36.7(4)	35.5(4)	37.5(5)	36.0(5)
F.	36.5(5)	35.3(5)	37.6(5)	35.9(5)
M.	37.5(5)	37.1(5)	37.8(5)	37.0(5)
M.	36.7(5)	35.0(5)	38.2(4)	36.9(4)
F.	37.5(4)	36.1(4)	38.3(5)	37.1(5)
M.	36.9(4)	35.5(4)	37.0(5)	36.1(5)
Mean	37.0	35.8	37.8	36.6
Mean difference	1.2		1.2	

* Sodium pentobarbital (Nembutal).

** Numbers in parentheses indicate number of records averaged. Readings taken at approximately half-minute intervals.

In experiments which involved a male and a female deer mouse, brief attention was given to the effect of high air temperatures on body temperature. These mice were exposed to air temperatures ranging from 40° to 50° C. for varying lengths of time. These experiments indicated that the upper limit of body temperature which could be tolerated for any length of time by *Peromyscus leucopus* was somewhere between 42° and 43° C. Apparently such high body temperatures can be tolerated for only brief periods without permanent impairment of temperature regulation. Exposure to temperatures of 45° and above resulted rather rapidly, usually in the space of about one-half hour, in impairment of thermoregulation. No return to normal body temperatures took place after exposure to air temperatures of 45° to 50° C. Non-reversible upper lethal temperatures between 43° and 44° were obtained for both of the experimental animals. These results are in

general accord with those for the rat (Adolph and Lawrow, 1951) and for similar sized mice (Bodenheimer, 1949; Fuller and Hiestand, 1947).

Normal body temperatures. A series of esophageal temperatures was taken on several individual mice at non-stimulating air temperatures (Table I). The minimum or lowest reading obtained for each individual is considered to represent resting conditions, while the mean possibly indicates body temperatures of mice when in an active state and performing various routine activities in their natural environ-

TABLE III

Esophageal temperatures of Peromyscus leucopus noveboracensis in relation to air temperature with and without food and water

Mean air temperature (° C.)	Sex and number	Hour of Exposure											
		1	2	3	4	5	6	7	8	9	10	11	12
		Mean esophageal temperature with food and water provided											
−30	M 10b	30.5	20.1	10.2									
−30	F 7c	32.7	23.4	9.9									
−20	F 8c	38.7	38.3	38.4	38.2	38.1	38.0	37.9	38.0	38.2	38.1	38.0	38.1
−20	F 9c	38.0	38.1	38.2	38.4	38.5	38.2	38.0	38.1	37.9	37.7	37.8	37.9
−14	M 11b	37.6	37.6	37.8	37.4	37.8	37.9	38.0	38.1	38.1	38.0	37.9	37.9
−14	F 10c	38.0	38.1	38.1	38.2	37.9	38.1	37.8	37.9	37.8	38.0	37.9	38.0
− 1	M 12b	37.5	37.6	37 9	38.0	37.9	37.7	38.1	37.9	37.9	37.7	37.8	37.9
− 1	M 13b	38.1	38.2	38.0	38.4	38.3	38.5	38.2	38.1	38.0	37.9	38.0	38.1
25	F 11c	38.4	38.2	38.0	38.2	38.2	38.5	38.3	38.1	38.0	37.9	38.4	38.3
25	M 14b	37.9	37.6	37.7	37.4	37.7	37.8	37.7	37.6	37.4	37.8	37.9	38.0
		Mean esophageal temperature without food and water											
−30	M 15b	31.7	14.5										
−30	F 12c	36.3	16.8										
−20	F 8c	38.8	37.9	23.9									
−20	F 10c	37.6	37.1	20.1									
−12	M 11b	37.8	37.6	37.3	37.7	37.9	38.1	37.8	37.5	30.1	11.7		
−12	F 9c	38.1	38.0	38.5	38.1	37.9	38.3	38.3	32.2	14.6			
0	M 12b	37.8	37.9	37.6	37.3	37.1	37.0	38.0	37.7	37.5	37.5	37.4	37.4
0	M 13b	38.4	38.6	38.7	38.8	38.6	38.2	38.1	38.0	38.4	38.1	38.5	38.4
26	F 11c	37.9	38.1	38.3	38.0	38.0	38.5	38.0	37.9	37.8	38.1	38.1	38.2
25	M 14b	37.6	37.5	38.2	38.1	38.0	37.9	37.9	37.7	37.9	37.6	37.5	37.6

ment. The mean body core temperature seems to lie between 37° and 38° C. The body temperature of all female mice averaged 0.5° C. higher than that of males, although in a few cases individual means were lower. The complete range of variability of body temperature in the mice measured was from 2.1° to 4.5° C. with a mean of 3.0° in the females, and from 0.8° to 3.8° C. with a mean of 2.6° in the males. Variation in body temperature became very much reduced when the mice were quiet and body temperature fluctuations were then in the neighborhood of ± 0.5° C.

Esophageal and subcutaneous temperature differences. It is well known that gradients in temperature exist between the interior of the body and the surface or subcutaneous area, due to differences in rates of heat loss and heat production in these regions. The difference between subcutaneous and esophageal temperatures in white-footed mice was determined by alternately measuring esophageal and subcutaneous temperatures at approximately half-minute intervals with an indicator potentiometer. While at room temperature, temperatures were recorded from the

TABLE IV

Subcutaneous temperatures of Peromyscus leucopus noveboracensis in relation to air temperature with and without food and water

Mean air temperature (° C.)	Sex and number	Hour of Exposure											
		1	2	3	4	5	6	7	8	9	10	11	12
		Mean subcutaneous temperature with food and water provided											
−30	F 1c	36.3	26.4	10.7									
−30	M 1b	37.2	25.7	9.9									
−30	M 2b	32.4	28.5	5.3									
−25	M 3b	36.5	36.7	36.0	35.9	36.9	37.0	37.3	37.0	36.5	36.7	36.8	37.1
−24	F 2c	37.5	38.7	39.1	38.7	38.3	37.9	37.4	37.4	37.6	36.9	36.3	35.8
−24	M 4b	39.1	38.1	37.5	37.4	36.8	36.6	34.1	34.1	33.9	29.5	4.6	
−22	F 3c	35.8	37.1	38.6	38.6	38.3	37.9	38.2	37.8	36.6	35.4	26.3	4.1
−12	F 3c	38.4	38.2	38.0	37.9	38.2	38.6	38.5	38.6	38.1	38.0	38.1	38.2
−12	F 4c	37.7	37.8	37.6	37.8	37.9	37.9	38.0	37.8	37.7	37.7	37.9	37.8
26	M 1b	37.4	37.2	37.1	37.6	37.5	37.5	37.4	37.2	37.0	37.1	37.2	37.2
26	F 4c	37.9	38.0	38.0	38.1	38.3	38.1	37.9	37.8	37.7	37.8	37.6	37.9
		Mean subcutaneous temperature without food and water											
−30	F 5c	30.8	11.5										
−30	M 5b	29.7	9.8										
−20	F 6c	38.2	34.7	10.5									
−20	M 6b	37.3	28.6	10.2									
−18	M 7b	37.3	33.5	13.9									
−14	M 8b	33.9	33.5	33.9	33.5	36.6	34.3	11.9					
−12	M 9b	37.2	36.4	36.7	35.7	35.7	38.3	37.9	30.9	12.7			
− 1	M 6b	37.2	37.7	37.4	37.3	37.0	37.8	37.5	37.4	37.7	37.6	37.3	37.6
− 1	F 6c	38.4	38.0	38.1	38.3	38.2	38.0	38.5	38.3	38.4	38.1	38.1	38.2
28	M —	37.6	37.7	36.1	37.0	36.8	36.9	36.3	37.0	36.5	36.2	37.3	39.3
27	F —	37.0	36.9	37.3	37.2	37.7	37.7	38.3	38.6	38.1	37.9	38.0	38.0

same animals when under light anaesthesia and two hours after recovery from anaesthesia (Table II). Individual differences between esophageal and subcutaneous temperatures ranged from 0.4° to 1.7° for anaesthetized animals and from 0.9° to 1.7° for those which had recovered from anaesthesia. The mean difference between subcutaneous and esophageal of anaesthetized animals was the same as that of animals which had recovered from anaesthesia.

No simultaneous comparisons between esophageal and subcutaneous body tem-

peratures were made at low air temperatures, but some idea of the relationship between the two temperatures in active, unanaesthetized animals can be obtained by comparing individual determinations of esophageal and subcutaneous temperatures at different air temperatures (Tables III and IV). However, as the same individuals were not used in the two series of determinations, temperature differences cannot be determined for individual mice.

Body temperature in relation to cold stress. Both esophageal and subcutaneous temperatures were determined at different degrees of low temperature and both

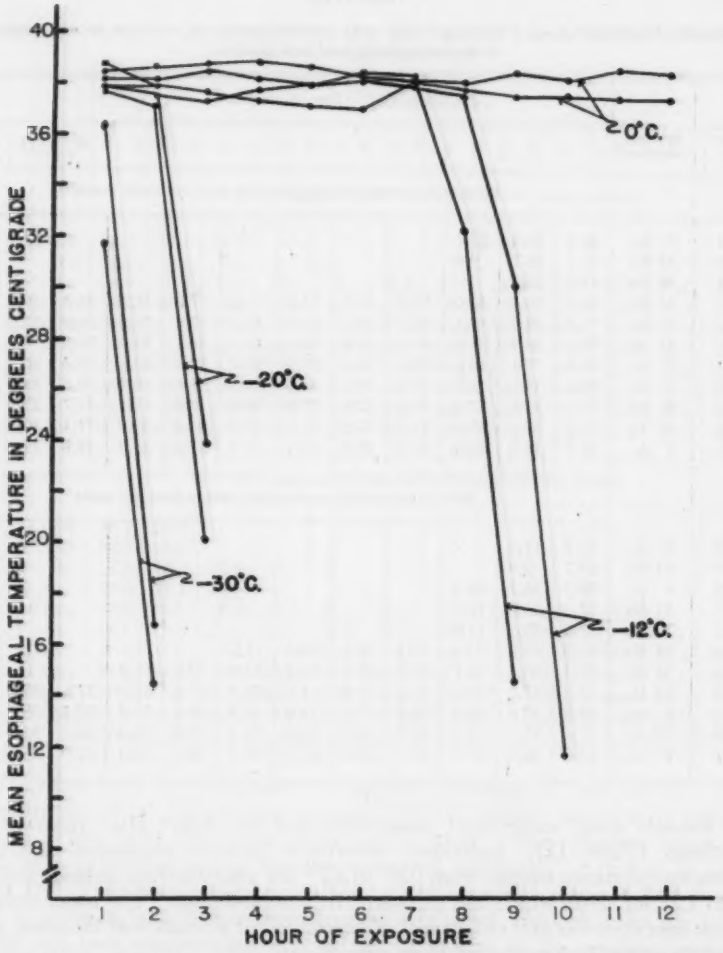

FIGURE 1. Mean esophageal temperatures of eight adult *Peromyscus leucopus noveboracensis* subjected to cold stress (0° C. to − 30° C.) when deprived of food and water.

with and without food and water. Continuous measurements of subcutaneous temperature were made, while esophageal temperatures were recorded within a 10-minute period outside the low temperature chamber after which the animals were again placed at low temperature. The possibility exists that this disturbance may have significantly altered the esophageal temperature readings. The esophageal temperature for each individual was determined at room temperature before subjecting it to lower temperatures. The mean temperature after the first hour of exposure was then averaged with the mean esophageal temperature at room temperature to give the mean temperature during the hour. The mean temperature at the end of each successive hour of exposure was then averaged with that at the end of the preceding hour in a similar fashion. Average subcutaneous temperatures were computed from automatically recorded subcutaneous temperatures for each hour of exposure. Plus and minus variations, which were of the order $\pm$ 0.5° C., tend to cancel each other out so that these averages are considered to be quite accurate.

Animals which were used more than once were kept at room temperatures for one to three days before exposing them to low temperatures again. None of the animals were exposed to low temperatures for more than 12 hours at a time, and those animals which were exposed to temperatures below 0° C. were held at room temperature for at least two days before they were again subjected to low temperature.

With but two exceptions, all individuals which were provided with food and water maintained quite uniform subcutaneous and esophageal temperatures during the entire 12-hour period of exposure at room temperatures down to about $-$ 25° C. At temperatures around $-$ 30° C. the mice in nearly all cases became hypothermic before the end of the first hour of exposure. When food and water were withheld the mice maintained constant temperatures during the 12-hour period only at air temperatures above 0° C. (Tables III and IV). Figure 1 shows the relationship between esophageal temperature, air temperature, and length of exposure at various degrees of low temperature with food and water withheld. Essentially the same relationship holds for subcutaneous temperatures of animals exposed to low temperature in the absence of food and water.

Some mice showed a very gradual rise in the mean body temperature during initial exposure to cold. This rise in body temperature was probably associated with an elevation of metabolism caused by exposure to low temperature as reported by a number of investigators (Adolph, 1950; Adolph and Lawrow, 1951; Benedict and MacLeod, 1929; Bodansky and Duff, 1936; Gosselin, 1949; Hart, 1950; Horvath *et al.*, 1938; Penrod, 1949; and others).

Discussion

When subjected to sufficiently low air temperature all mammals will eventually undergo a lowering of body temperature from which recovery is possible if the body temperature stays within the animal's tolerable range of hypothermia. The limits of this range vary with the duration and severity of the low temperature to which the mammal is exposed. Determinations of normal body temperatures and lower lethal temperatures for several species of mammals (Haterius and Maison, 1948; Luyet and Gehenio, 1940; Scott and Bazett, 1941; Wislocki, 1933) show

that the tolerable range of hypothermia is often wide, ranging from about 13° to 37° C. For most species of mammals, including the white-footed mouse, *Peromyscus leucopus*, the tolerable range is somewhere between 20° and 25° C.

The lower lethal limits of body temperature in different species of rodents vary considerably. Hart (1951a) recorded lethal colonic temperatures from 11 deer mice, *Peromyscus maniculatus*, which ranged from 0.6° to 13.9° C. with an average of 9.2° C. The mean lethal temperature of 4.6° C. for *Peromyscus leucopus* determined in this study is considerably lower although the criteria of lethality may have varied. A Palestinian mouse, *Meriones tamaricinus*, was found to have a lower lethal temperature of about 13° C. (Bodenheimer, 1949). Lethal body temperatures of 8 white mice averaged 10° C. (Hart, 1951a). Lower lethal limits for the white rat (Adolph, 1948) and the guinea pig (Gosselin, 1949) are somewhat higher than for mice, while that of the hamster (Adolph and Lawrow, 1951) is considerably lower.

Species differences and differences in methods of measuring the lethal point undoubtedly account for much of the variation in lower lethal body temperatures of similar sized rodents. Other factors which may alter the lethal temperature are sex, amount of activity, nutritive state, relative humidity and previous acclimation to high or low temperatures. Thus the range of tolerance may vary to some extent depending upon the conditions of the experiment.

The range of tolerance above average body temperatures for most of the smaller species of rodents appears to be about the same. Data on normal body temperatures and upper lethal temperatures for the rabbit and dog (Heilbrunn, 1943; Wislocki, 1933) give tolerable ranges of hyperthermia from 4.0° to 7.5° C. The range is somewhat wider for the echidna and sloth (Britton and Atkinson, 1938; Enders and Davis, 1936; Martin, 1902, 1930; Morrison, 1945; Sutherland, 1897) in which it is between 5° and 9° C.

It seems evident from the present study that an adequate supply of food and water is important in avoidance of hypothermia at low temperatures. In addition, behavior responses such as huddling, burrowing and nest-building enable small mammals to avoid hypothermia at very low ambient temperatures (Kinder, 1927; Richter, 1927, 1942; Scholander, Walters, Hock and Irving, 1950; Sealander, 1952).

Behavior adjustments are possibly of even greater importance in avoidance of hyperthermia at high ambient temperatures. These include postural adjustments to expose greater body surface, spreading of saliva on the fur (Herrington, 1940, and this study) and decreases in amount of motor activity. According to Sumner (1925) small desert mammals have no special ability to resist high temperatures but are nocturnal in activity and remain in their burrow system during the hotter part of the day. Adjustments of this sort are more easily made than changes in the animal's energy balance.

Summary

1. Observations were made on behavior responses and body temperatures of white-footed mice, *Peromyscus leucopus noveboracensis*, subjected to ambient temperatures ranging from − 35° C. to + 50° C.

2. Behavior responses to low temperature included gnawing, grooming movements, running, rapid breathing and shivering. Motor activity was greatest upon

initial exposure to cold and at moderately low temperatures (0° to − 20° C.) and decreased almost entirely at lower temperatures (− 20° to − 35° C.). Behavior responses to high temperatures involved a decrease in activity and assumption of postures which exposed the maximum body surface area. Salivation occurred quite freely at temperatures between 40° and 50° C.

3. The lowest minimal recoverable body temperature observed was 10° C. This was an esophageal temperature when the mouse was exposed to air temperatures ranging from − 23° C. to − 12° C. over a period of 65 minutes. Activity was minimal during the entire period of exposure.

4. The mean lower lethal body temperature (esophageal) was 4.6° + 0.5° C. (15 determinations). Lower lethal body temperatures ranged from 3.5° to 5.0° C. The lower lethal body temperatures were determined at ambient temperatures ranging from − 12° to − 35° C. and the duration of exposure before death occurred varied from 45 minutes to 8.5 hours. There was, however, no apparent correlation between the lower lethal body temperature and the duration of exposure or ambient temperature. Upper lethal body temperatures of two mice were between 43° and 44° C.

5. At room temperatures esophageal temperatures ranged from 35.5° to 40.5° C. with a mean of 38.1° C. for males, and from 35.4° to 40.5° C. with a mean of 37.6° C. for females. The difference between sexes may not be significant.

6. Differences between esophageal and subcutaneous body temperatures of individual mice ranged from 0.4° to 1.7° C. The mean difference of 1.2° C. for seven mice remained the same under light anaesthesia although both esophageal and subcutaneous temperatures were subnormal.

7. Body temperatures of mice supplied with food and water remained rather constant when the mice were subjected to cold stress, except at ambient temperatures around − 30° C. When food and water were withheld all mice became hypothermic within 12 hours at all temperatures below − 1° C. These findings indicate that ordinary low temperatures may not be critical for small mammals in nature unless they are combined with lack of food, water and possibly shelter. Behavior responses such as huddling, burrowing and nest-building may give enough additional protection to offset most of the adverse effects of low air temperatures.

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CHEMOTROPISM IN RHIZOPUS NIGRICANS. II. THE ACTION OF PLANT JUICES

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Parasitic fungi invade the tissues of their host plants by sending their growing hyphal tips through the stomata of the leaves or by actually piercing the epidermis. To pass through a stoma, the hypha must first grow to a particular point on the leaf surface. To penetrate the epidermis, it must break through a barrier. Investigators have long believed that such non-random patterns of growth must involve some directive influence by the host plant. Such influence seems even more necessary in view of the high specificity shown by some parasites—they will invade the tissues of only a particular kind of host. The question of how a host plant could direct the growth of its parasite led to some early studies of chemical tropism in fungi.

The first work dealing directly with this problem was that of Miyoshi (1894), in which he sowed spores of several molds on the under surface of Tradescantia leaves which had been injected with various chemical substances. These substances presumably diffused out through the stomata and onto the surface. He reported that when the leaves contained proper concentrations of certain nutrients (particularly sugars and meat extracts), the hyphae grew directly to and through the stomata. On leaves injected only with water, he found the hyphae to grow in random directions, while if the leaves contained harmful substances (acids, alkali, alcohol and certain salts), growth was oriented away from the stomata. He observed the same tropic responses when he sowed the spores on mica plates with pin holes in them, the substance under test being in a gelatin layer in contact with the opposite face of the plate. Thus Miyoshi concluded that fungi exhibit an extensive (and extremely useful) chemotropic sensitivity, guiding them to supplies of nourishment and away from damaging materials.

The case for chemotropic control of parasitic molds was expanded by Massee (1905). He confirmed Miyoshi's finding that various fungi grow tropically toward sugars. But he found obligate parasites to be attracted only by decoctions from their own hosts. He believed facultative parasites to be attracted by sugar, but repelled by certain other substances, which prevented them from invading certain types of hosts (thus he said Botrytis failed to grow into green apples because of a negative tropic sensitivity to malic acid). Massee concluded that the specificity of parasitism was based completely on chemotropism. His findings have not been confirmed in any other work, to our knowledge. Certain substances for which he reported strong chemotropic action have been tested on Rhizopus in the course of the current study, but always with negative results.

The findings of Miyoshi were checked by Clark (1902), using the injected Tradescantia leaves, but with quite different results. He wished particularly to test the suggestion of Swingle (1896) that sprays of copper compounds protect host

plants through a negative chemotropic action on parasitic fungi. He concurred with Miyoshi in the finding that the hyphae grew into Tradescantia stomata toward sugars, but he found them to grow in just as readily if the leaf had been injected with plain water or even with toxic copper salts. Likewise, on mica plates, the hyphae grew into the hole regardless of what substance was on the other side. The only condition which prevented growth toward the hole was the presence of germinating spores in the layer on the other side. He concluded that the germ tubes growing toward the holes when no spores were present on the other side must have tropic sensitivity to some product of the mold metabolism and must be growing *away* from higher concentrations of this material. This growth behavior has been called the "staling reaction," as it is growth away from regions of stale medium. Clark's conclusion that molds have a negative tropic sensitivity to some product of their own metabolism has been adequately confirmed by later studies (Fulton, 1906; Graves, 1916; Stadler, 1952).

There has been some disagreement as to whether sugars and other nutrients exercise a positive chemotropism on molds. Miyoshi (1894) reported pronounced turning of the hyphal tips toward concentrations of various nutrients, but Clark (1902) maintained that the oriented growth was caused only by the negative tropic effect of the staling factor, and that hyphae could grow just as readily into water or harmful substances as into nutrients. This was the view also of Fulton (1906), who looked for tropic action by a large number of substances on eight different molds; he detected no positive tropism and concluded that orientation was based solely on the staling reaction. Graves (1916) recognized that the staling reaction caused the most marked orientation, but he reported that hyphae turned more pronouncedly toward a sugar-containing medium than toward plain water; thus, he concluded that sugar does exercise an attraction, but that it is normally masked by the stronger tropic action of the staling substance.

Some of the experiments on sugars were repeated during the present study in an attempt to determine whether or not there was any tropic action by these substances. The interpretation of such studies is complicated by the fact that staling-substance production (and thus orientation caused by the staling reaction) varies with sugar concentration. When the experiments were designed in a way to minimize variation in the staling reaction, the results gave some indication of a very slight attraction by glucose and by sucrose, but they were not conclusive.

Graves reported that turnip juice medium had a much more powerful positive tropic effect than sugars, though again, not as strong as the staling reaction. The turnip juice finding was reminiscent of the report of Massee (1905) and other early suggestions that tropic attraction by plant juices might be responsible for host penetration by parasitic fungi.

In the present study the tropic action of turnip juice and other plant materials on germinating spores of *Rhizopus nigricans* has been extensively studied. These materials cause striking orientation, but further investigation of this phenomenon has led us to the conclusion that this orientation is not the result of a direct tropic action on the mold by the plant juices.

Methods

The method used in most of the experiments is the same as described previously (Stadler, 1952); plates of plastic coverslip material with circular holes drilled in

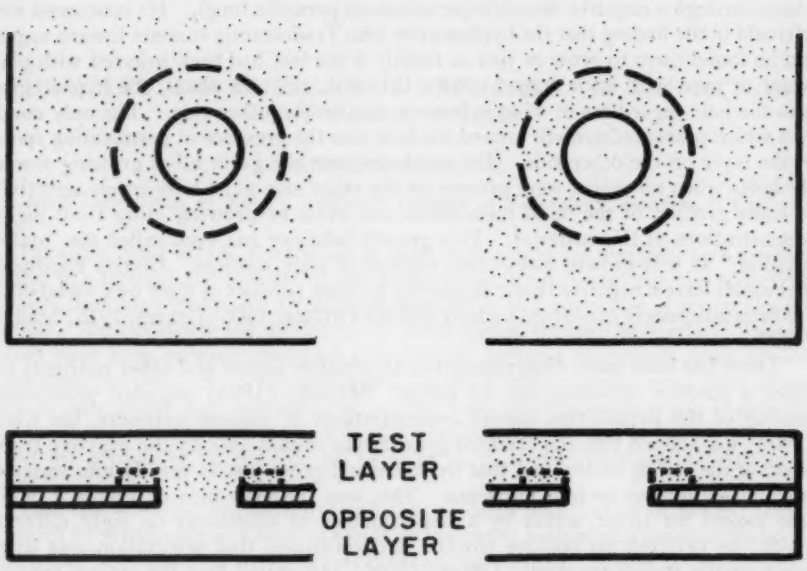

FIGURE 1. Enlarged representations of the corner of a plate showing one hole in face view and in profile. On the left is the plate as prepared in the ordinary tests. The dots represent the spores of the test layer, and the broken line shows the limits of the test region. The picture on the right shows a preparation made by the "thumb tack" method, whereby no spores are present within or above the hole and the test region becomes the edge of the population.

them are used. A layer of agar medium is placed on each side of the plate; the agar layers are in contact with each other only within the holes. Any material contained in one layer but not in the other will tend to diffuse through the hole, setting up a concentration gradient in the region in and near the hole. One of the layers, in every case, contains a suspension of spores of Rhizopus. This is the "test layer." The direction of growth is studied of the spores in the test layer in the region immediately around the hole. The other layer is called the "opposite layer" and may or may not contain spores, depending on the experiment being done. The preparations are incubated for eight and one half hours at 28° C. and then fixed and stained. Camera lucida drawings are made of the spores in each of the "test regions" (washer-shaped region circumscribing the hole, Fig. 1), and the directions of growth are measured. The angle measured is the direction of growth of the germinating spore with respect to the direction of the chemical gradient. Any gradient set up between the two layers must be oriented directly out from the center of the hole. When a germ tube grows directly toward the center of the hole, the angle recorded is zero; when it grows directly away from the hole, the angle is 180 degrees.

If the germ tubes of spores in a particular experiment are growing in random directions, then the average of the angles for all the spores in a test region should be about 90 degrees. This is the result when we do an experiment with spores in nu-

trient medium in the test layer and the same concentration of spores in the same medium in the opposite layer. (The synthetic medium used in these experiments contained 1% glucose, 10^{-2} *M* aspargine, 10^{-2} *M* KH_2PO_4, and 2×10^{-3} *M* $MgSO_4$.) When a plate is prepared with spores in the nutrient medium in the test layer, and the opposite layer is made up with the same medium (or even water agar) but with no spores, then the spores in the test regions grow toward the holes. If the test layer contains 120 spores per mm.^{3}, the average angle will be 40–45 degrees (Fig. 2-A). This is the staling reaction—growth away from populated regions, away

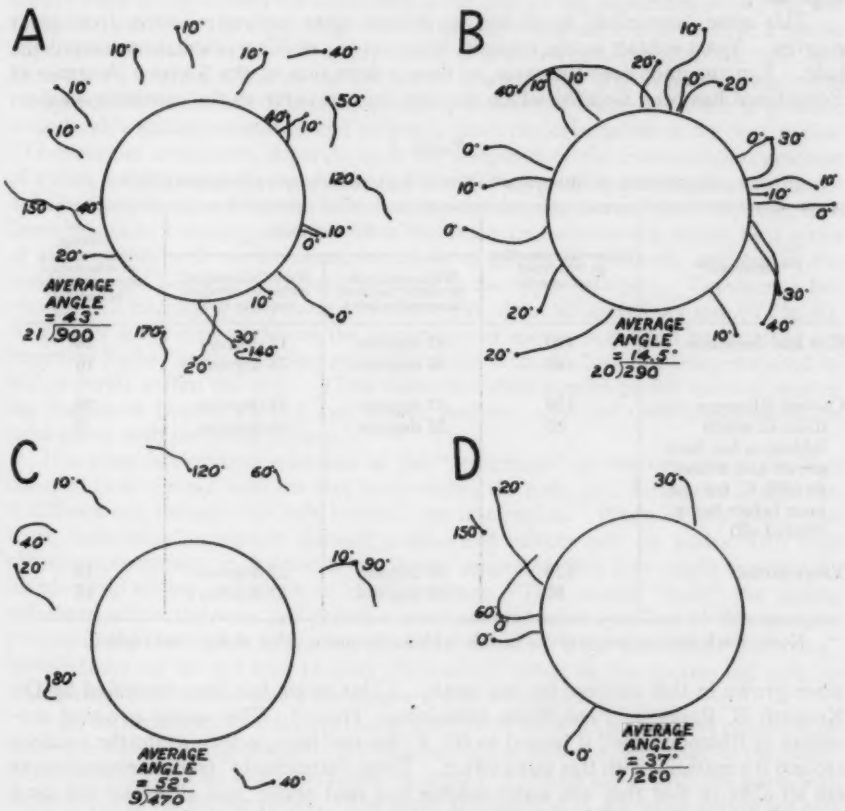

FIGURE 2. Camera lucida drawings of test regions from experimental plates showing the improvement of orientation elicited by plant juices in the opposite layer. *A:* 120 spores per mm.^{3} in the synthetic medium in the test layer, non-spore synthetic medium in the opposite layer; *B:* 120 spores per mm.^{3} in synthetic medium in the test layer, non-spore elm leaf decoction in the opposite layer; *C:* 40 spores per mm.^{3} in synthetic medium in the test layer, non-spore synthetic medium in the opposite layer; *D:* 40 spores per mm.^{3} in the synthetic medium in the test layer, non-spore elm leaf decoction in the opposite layer.

from concentrations of the unknown metabolic product (or products) called the staling substance.

The "Attraction" of Plant Juices

If turnip juice is mixed into the non-spore opposite layer of a plate with spores in synthetic medium in the test layer, all the spores in the test region will germinate and grow toward the hole. The orientation is much more marked than when synthetic medium is used in the non-spore opposite layer (Fig. 2-A, B); in that case the average angle was 40–45 degrees; with turnip juice in the opposite layer it is 10–20 degrees.

This same "attraction" is elicited by several other natural mixtures from plant sources. Yeast extract in the opposite layer causes striking orientation toward the hole. Tomato juice does the same, as does a decoction of elm leaves. A strain of Penicillium has been isolated which imparts this property to the synthetic medium

Table I

Comparison of "attraction" effect in high and low spore concentrations

"Attractant"	Spore concentration in test layer (per mm.[3])	Average Angle		Average improvement of orientation (in degrees) by "attractant"
		With non-spore synthetic medium in opposite layer	With "attractant" added to nonspore opposite layer	
Elm leaf decoction	120	45 degrees	17 degrees	28
	40	54 degrees	38 degrees	16
Cooked Rhizopus (medium in which Rhizopus has been grown and heated to 60% C. for one hour before being filtered off)	120	47 degrees	27 degrees	20
	40	55 degrees	46 degrees	9
Yeast extract	120	42 degrees	22 degrees	20
	40	57 degrees	42 degrees	15

Note: Each average angle given in this table is the mean value of four test regions.

when grown in that medium for one week. (This strain has been identified by Dr. Kenneth B. Raper as *Penicillium spinulosum* Thom.) The young growing mycelium of Rhizopus itself if heated to 60° C. for one hour, releases into the medium around it a material with this same effect. These "attractants" from various sources are all alike in that they are water-soluble and heat stable, and all evoke the same marked orientation of Rhizopus on the plates used in this study. Furthermore, they are alike in that there is evidence in each case that the material does not exercise a true chemotropic attraction on the Rhizopus spores.

The suspicion arose that these materials might not exert a simple attraction on Rhizopus when it was noted that a preparation which markedly improved the orientation on plates with high concentrations of spores in the test layer had much less effect on orientation on plates with low spore concentrations (Table I, Fig. 2).

This suggested that the action of these materials might be somehow related to the staling reaction, since the intensity of this reaction varies with spore concentration. If the material were a true attractant, it should act on spores in its presence regardless of the intensity of the staling reaction.

Orientation, in these experiments, is normally limited by a shortcoming of the method. The strongest orientation elicited by the staling reaction should be shown by the spores on the *edge* of the populated region, as this is where the concentration of staling substance is diminishing most markedly. The spores in the test regions of these plates are not on the edge of the population, but near the edge. There are always some spores *within* the hole—this is the edge of the population mass; the test region is back from the edge (Fig. 1). When an experiment is done with a sparse population of spores in the test layer, there are very few spores within the hole, and these have a minimal effect on the amount of orientation. But in heavy populations the spores within the hole become important, as there are enough of them to produce considerable staling substance and seriously limit the orientation in the test region. The extent of orientation depends upon the steepness of the concentration gradient of staling substance in the test region. If it is being produced in large amounts not only on the side away from the hole, but also on the side toward (in) the hole, then there cannot be a sharp gradient. That this is the true situation is shown by a series of experiments with various concentrations of spores in synthetic medium in the test layers and non-spore synthetic medium in the opposite layers. Orientation improves with increasing spore concentration up to about 80 spores per mm.[3] (Fig. 3). At greater spore concentrations the orientation of spores in the test region does not improve; higher levels of staling substance away from the hole are being matched by higher levels within the hole. (The same limitation applies to the method used in the studies of Graves (1916) and earlier workers, who did their experiments on mica plates with pin holes in them.)

If a plate is prepared with one of the "attractants" in the opposite layer, this material is in contact with the test layer within the hole, and during the incubation it diffuses out through the hole towards the test region. When it was found that these "attracting" materials showed pronounced effects only on plates with high spore concentrations in the test layer, it was suspected that they might function by inactivating staling substance in their presence. This would "flush" the staling substance within the hole, and permit a steep concentration gradient of this material to arise in the test region. The hypothesis is advanced, then, that these "attracting" materials act not by any true positive chemotropic effect on the spores, but only by inactivating a substance which does have a true tropic action.

As a further check of this hypothesis, test plates were set up with no spores in or above the hole. In this situation the test region becomes the very edge of the population (Fig. 1). This is accomplished by means of thumb tacks with the same bore as the holes in the plastic plates. The tacks are inserted in the holes before the test layer is poured, and after it has solidified they are removed, leaving a cylindrical hollow above each hole. When the plate is placed on the liquid, non-spore agar medium of the opposite layer, this material fills these hollows before it solidifies. When the growth on these preparations is examined, the test spores are found to show excellent orientation (15–20 degrees average), regardless of whether an "attractant" is present in the opposite layer or not. Thus the pronounced effect of

the "attractants" seems to be directly dependent on the presence of spores within the hole.

A simpler test which indicates that this is not a true attraction is done with a layer of agar medium on a glass slide. Spores are inoculated only at one end, and the hyphae grow down the length of the slide. A block of the synthetic medium agar in the region ahead of the advancing hyphae is cut out and replaced by a block of turnip juice agar. If it were a true attractant, hyphae passing nearby should turn and grow into it, but they do not.

The experiments so far described fit very well the hypothesis that the "attracting" materials act only by inactivating staling substance in their presence. However,

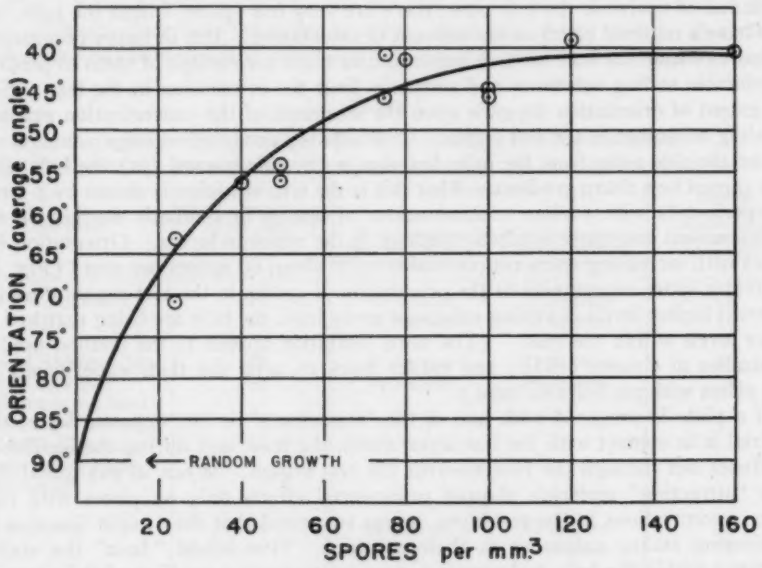

FIGURE 3. Orientation plotted against spore concentration for experiments with spores in synthetic medium in the test layer and non-spore synthetic medium in the opposite layer. Each point represents the average of four test regions.

with undiluted tomato juice agar in the opposite layers of test plates, we observe marked improvement of orientation even when there is a low concentration of spores in the test layer. The method used in this experiment is not satisfactory in that it can never tell us whether the improved orientation results from an enhanced staling reaction or from a true positive tropic effect. This is because both of these possible effects would be working in the same direction in this system, and there is thus no visible way to distinguish between them. The same ambiguity exists, to a limited extent, in the preparations with no spores within the holes, though these were designed to minimize any effect on the staling reaction. The same is true for the experiment with a block of turnip juice agar in the neighborhood of growing hyphae.

A definitive test to learn the true nature of the effect can be designed by preparing the experiment in such a way that the "attractant" will be working in a direction antagonistic to the staling reaction. This is accomplished by putting the "attractant" material in the test layer with the spores, while the opposite layer contains non-spore synthetic medium. In this situation, the staling reaction tends to make the spores in the test region orient toward the hole, while the "attractant" tends to orient them away from the hole. If the experiment is prepared so that the staling reaction is very weak (low spore concentration), and the attraction is very strong (undiluted tomato juice), then we can tell whether it is a true attractant or a staling substance inactivator. If it is a true attraction, then it should completely outweigh the staling reaction in this situation and cause marked orientation away from the hole. If it works only by inactivating staling substance, then the most it can do is obliterate the staling reaction and cause random growth; 90 degrees becomes the limiting average angle. Table II shows the latter to be the case. With concentrated tomato juice on a series of plates with very low spore concentration, the average angle is below 90 degrees. With turnip juice, the result is the same. This is

TABLE II

Orientation of spores in plant juice with synthetic medium in the opposite layer

"Attractant" in test layer	Spore concentration in test layer (per mm.^{3})	Number of test regions studied	Total no. of hyphae	Total angle	Average angle
Tomato juice	25	16	74	6000 degrees	81 degrees
Turnip juice	35	15	105	8950 degrees	85 degrees

Note: In the experiments reported in this table, all opposite layers contained non-spore synthetic medium.

good evidence, then, that tomato juice and turnip juice, which showed the most marked effects of any of the materials in the previous tests, are not true chemotropic agents.

Attempts at purification of the active material by extraction of yeast extract with a number of solvents (methanol, propanol, butanol and chloroform) have been unsuccessful. Paper chromatograms of yeast extract with a butanol-acetic acid mixture as the moving solvent affected some separation of components but did not result in any appreciable purification of the active material. Further chemical studies are projected with other solvents and perhaps other of the source materials.

The presence of a dialysis membrane between the two agar layers on the test plate does not hinder the effect of the "attractant" (from the Penicillium medium), but there is indirect evidence that the active material is slow-diffusing. The "attraction" phenomenon is qualitatively unchanged at any acidity permitting growth of the mold (pH 3 to pH 7).

DISCUSSION

The experiments which were done to determine the nature of the action of plant juices on orientation of the growth of Rhizopus spores demonstrated that the in-

tensity of the plant juice effect varies with the amount of growing mold in its presence. The hypothesis has been advanced that the plant juices affect orientation by inactivating staling substance in their presence. It is also possible that they act by preventing the production of staling substance. A third possibility which fits the experimental findings equally well is that a true positive tropic agent is formed by the combination of a plant juice ingredient with some product of the mold's metabolism. Not knowing the chemical nature of the active plant juice material, it is difficult to design experiments which would discriminate between these various hypothetical mechanisms.

The present study does not enable us to evaluate the significance in nature of the tropic action of plant juices. Although these materials do not exercise direct attraction on the hyphae (of Rhizopus, at least), they do act in a way which could facilitate entrance into the stomata by germ tubes of spores germinating on the leaf surface. To understand the part played by chemical tropism in host-parasite relationships, a careful study should be made of the tropic sensitivities of a series of parasitic molds. If their tropic responses are similar to those of Rhizopus, then it does not seem possible that host specificity could be based on unique chemotropic sensitivities, as suggested by Massee (1905). In view of the many instances of specific growth factor requirements of molds which have been demonstrated in recent years, it appears more probable that this is the basis of host-parasite specificity.

Summary

Turnip juice and several other plant materials exert what appears to be a strong tropic attraction on the germinating spores of *Rhizopus nigricans* when tested on the type of experimental plates used in this and earlier studies. However, evidence is presented which demonstrates that the plant juices do not exercise a direct tropic action on the mold. It is suggested that these materials function by inactivating the staling substance (a negative tropic agent which is a normal product of the mold's metabolism).

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RESPIRATORY GRADIENTS IN TUBULARIA[1]

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The presence of a respiratory gradient along the stem of Tubularia has been known for some time (Hyman, 1926; Barth, 1940a). It has been suggested that it is the underlying factor of the regeneration gradient (Barth, 1940b). One can easily find considerable evidence in previous works of various investigators in favor of this idea (Barth, 1940b). For instance, regeneration rates along the stem are roughly proportional to the rate of oxygen consumption and oxygen is an important factor in the rate of regeneration and in determination of the polarity of the stem. However, the evidence so far does not seem critical enough for its establishment.

More recently, Rose and Rose (1941), Miller (1942), and Goldin (1942a) have shown that a certain inhibitor (or inhibitors) of regeneration is produced by the stem. Goldin (1942a, 1942b) and Barth (1944) showed that a ratio involving the concentration of inhibitors and oxygen, $O_2/(\Sigma I)$, somehow determines the rate of regeneration and the polarity of the stem. Thus, another hypothesis suggested by Barth (1944) is that a gradient in the concentration of the inhibitor is the cause of polarity and of the determination of the polarity of the stem. Oxygen merely shifts the threshold of inhibition.

The purpose of the present investigation is to test which of the two hypotheses is more likely. It has been known that the regeneration polarity of Tubularia can be reversed by various methods. If the respiratory gradient is the cause of this polarity, one would expect that when we use one of the methods to reverse the polarity of the stem, the respiratory gradient should reverse its direction before the polarity.

Methods

There are a number of techniques which can be used for reversing the polarity of Tubularia, for instance, differential concentration of oxygen at two cut ends, ligature (Barth, 1938) etc. The method adopted for reversing the polarity in the present paper was ligature. The material used was freshly collected from Atlantic Beach, Long Island, New York City. A number of healthy stems was carefully selected. They were cut into fragments a little more than 10 mm. long. The distal ends of these fragments were then tied individually with a length of hair. Finally they were placed in sea water through which pure oxygen was bubbled, and kept at 15° C. until the initiation of measurements.

For measuring the oxygen uptake, Cartesian diver technique was used. Before the measurement of O_2 uptake, fragments were individually checked under a microscope to see whether they were in healthy condition and whether the primordia had

[1] Aided by a grant from the Committee on Growth acting for the American Cancer Society, administered by Dr. L. G. Barth.

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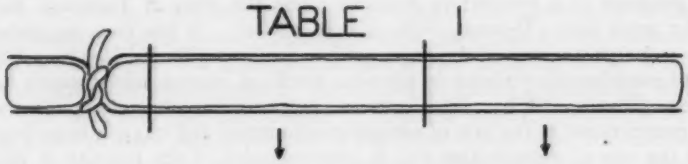
been formed. Only the healthy fragments were used in the experiments. Each fragment was then cut into two pieces, the distal and proximal halves of approximately similar size (Tables I, II and III). The respiratory rates of the two pieces derived from the same fragment were then compared. All measurements were made at 25° C. It is very hard in New York City to keep the temperature below this in summer. Although this temperature is higher than normal, the rate of respiration during the period of measurement is constant in all cases. After the measurement, the pieces were carefully taken out of the divers. They were rinsed



Frag. No.	Rate Distal	Rate Proximal
1	0.76	0.60
2	0.83	0.56
3	0.76	1.0
4	0.63	0.53
5	1.0	0.85
$\bar{X}$	0.80	0.71

TABLE I. The respiratory rates of the fragments of the non-regenerating stems. The rate $\times 10^{-3}$ µl./µg. dry wt./hr.

once with distilled water and were subsquently transferred onto the weighing pans. Following this, they were dried overnight in an oven at 120° C. and were then weighed on a quartz torsion balance.

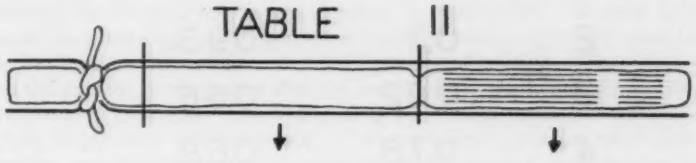
RESULTS

In Table I, there are five experiments made on three-day old fragments that have no primordium. Four fragments show that the distal half has a higher respiratory rate than the proximal portion. One fragment gives the opposite result. On the average the distal half respires more than the proximal. These results agree with the findings of Hyman (1926) and Barth (1940a).

Four experiments have been made on the fragments which have primordia at their proximal ends and no primordia at the distal ends (ligature end). The results are summarized in Table II. From the table one can see that all the distal halves invariably show higher respiratory rates than the proximal ends, although the latter bear primordia.

In Table III, there are five experiments in which the fragments have very well-developed hydranths. The measurements were made three days after the emergence of the hydranth from the perisarc. The pieces for measurements of oxygen uptake were cut in the way shown in the figure of Table III. Only the region that was covered by the old perisarc was used for these measurements. This method of cutting

TABLE II



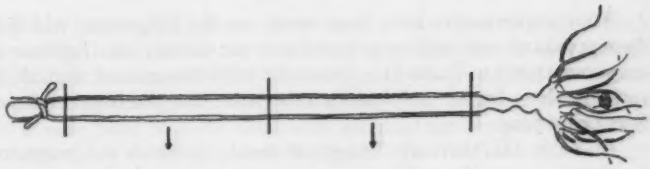
Frag. No.	Rate Distal	Rate Proximal
1	1.8	1.0
2	1.2	0.95
3	1.2	0.68
4	1.6	1.2
$\bar{X}$	1.5	0.96

TABLE II. The respiratory rates of the fragments of the regenerating stems. The rate $\times 10^{-3}$ μl./μg. dry wt./hr.

was employed in order to eliminate possible variation of the thickness of the newly formed perisarc from the old one. From the table we can see that there is no apparent difference between the distal and the proximal piece.

From the results, it is clear that before or during the process of regeneration, there is no apparent change in respiratory gradient. Unfortunately, no running sea water was available. Although sea water was changed every day during the course of investigation, the regenerated hydranth usually dropped off the stem on the third or fourth day after emergence of the hydranth from the perisarc. However, the results on the fragments with well developed hydranths do suggest the tendency of the reversal of the respiratory gradient, since the distal and proximal pieces of such fragments respire at approximately the same rate, three or four days

TABLE III



Frag. No.	Rate Distal	Rate Proximal
1	0.99	0.71
2	0.71	0.93
3	0.62	0.66
4	0.78	0.68
5	0.86	0.86
$\bar{X}$	0.79	0.77

TABLE III. The respiratory rates of the fragments of the polarity-reversed stems. The rate $\times 10^{-3}$ μl./μg. dry wt./hr.

after emergence of hydranth from perisarc. Probably, if one could keep the fragments long enough their respiratory gradient would eventually be reversed.

DISCUSSION

There are two drawbacks concerning the present results. First, the temperature at which the experiments were made may have been too high, even though constant respiratory rates were obtained in all cases during the period of measurement. Second, although measurements of two contiguous pieces of each fragment were made, as shown in the tables, there may nevertheless have been some variation in the thickness of the perisarc, a factor which would affect the total dry weight. In other words, the respiratory gradient could possibly be due to a posterior segment containing more perisarc material and less tissue. Some preliminary experiments indicate that this might well be the case. In the following discussion we shall assume that these two drawbacks are not of great importance. A more extensive study under controlled conditions is of course necessary.

It is possible that techniques which involve keeping an animal at one temperature and measuring oxygen consumption at another may produce a differential acceleration in regions which are compared. If there is any such differential effect of temperature, it does not appear to be important. Barth (1940a) prepared his material at room temperature and measured the oxygen uptake at 18.5° – 19.0° C. His results on the respiratory gradient of the stem are in agreement with the present findings in the experiments on the stems with or without primordia.

A number of investigators have demonstrated the presence of an inhibitor of regeneration. Certain experiments have indicated that the inhibitor substance is a fairly diffusible one (Rose and Rose, 1941; Goldin, 1942a). Barth (1940a) found that the O_2 consumption of regenerating stems is no greater than the O_2 consumption of resting, non-regenerating stems. Thus, stems which were prevented from regenerating by means of ligatures at the ends consumed as much oxygen as stems which regenerated freely. These facts suggest two things: (1) the inhibitor of regeneration is either a weak respiratory inhibitor or an inhibitor not affecting respiration at all; and (2) ligatures have no or very little effect on the respiration of the stem.

It was shown in the present investigation that prior to, or during the process of regeneration, the original respiratory gradient is maintained. In view of the conclusions in the preceding paragraph, these findings really represent what had been going on in the stems before removal of the ligature and cutting. Therefore one may say from the present investigation that the formation of the hydranth is independent of the respiratory gradient. The hydranth is not always formed at the place where the highest respiratory activity exists. If a respiratory gradient were involved in regeneration, one would expect, at least during the time of regeneration, that the primordium would show a higher respiratory activity than the distal segment in the present experiments. Since the presence of an inhibitor of regeneration in the stem has been demonstrated and it has also been found that oxygen plays a very important role in regeneration, it seems most likely that the position of a future hydranth in a regenerating stem is determined by the balance between the concentration of the inhibitor of regeneration and the concentration of oxygen.

Finally, the present findings seem to suggest that the respiratory gradient of the stem is the result of the presence or the activity of a well developed hydranth.

Summary

It was found that during regeneration of the hydranth, the original respiratory gradient of the stem is maintained. Only after the hydranth has formed has the stem a tendency to reverse its original respiratory gradient.

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